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(54) Title: TREATMENT OF CYSTIC FIBROSIS

(57) Abstract: The present invention relates to methods of treating cystic fibrosis, and in particular to exposing cystic fibrosis cells to alkalinizing agents.

## TREATMENT OF CYSTIC FIBROSIS

This application claims priority from pending United States patent application Serial Number 60/254,712, filed December 11, 2001.

5 This invention was made in part during work partially supported by the U.S. National Institutes of Health grant AI 31139. The United States government may have certain rights in the invention.

### FIELD OF THE INVENTION

10 The present invention relates to methods of treating cystic fibrosis, and in particular to exposing cystic fibrosis cells to alkalinizing agents.

### BACKGROUND OF THE INVENTION

Cystic fibrosis is a genetic disease affecting approximately 30,000 children and  
15 adults in the United States. More than 10 million people (one of every 31 Americans) are unknowing, symptom-free carriers of the disease. The disease develops in individuals who inherit a defective copy of the cystic fibrosis gene from each parent.

The cause of cystic fibrosis is a mutation in the gene for cystic fibrosis transmembrane conductance regulator (CFTR), which functions as an apical membrane  
20 chloride channel (Schwiebert E. M., *et al.* (1999) *Physiol Rev* 79, S145-66; Bradbury, N.A. (1999) *Physiol. Rev* 79: S175-91). Various CFTR mutations in cystic fibrosis affect the processing, intracellular localization, and function of this protein (Schwiebert E. M., *et al.* (1999) *Physiol Rev* 79, S145-66; Bradbury, N.A. (1999) *Physiol. Rev* 79: S175-91), as well as affect the function of other ion transporters. The result of these  
25 mutations is the production in the body of an abnormally thick, sticky mucous, due to the faulty transport of sodium and chloride within the cells lining organs, such as the lungs and pancreas. A common symptom is coughing, wheezing, and pneumonia.

Treatment of cystic fibrosis depends upon the stage of the disease, which organs are involved, and how they are affected. Many treatments are directed to alleviating the  
30 symptoms due to the presence of the mucous; for example, chest physical therapy, which requires vigorous percussion (by using cupped hands) on the back and chest, is used to dislodge the thick mucous from the lungs. Other treatments involve the delivery of drugs to the lungs to break up the mucous.

Other treatments are directed to treating the cause of the disease. On such promising treatment of cystic fibrosis is the development of gene therapy, in which normal CFTR genes are transformed into the lung cells of cystic fibrosis patients; currently, about 180 individuals have been experimentally treated with gene therapy. However, this  
5 treatment suffers from several drawbacks; these include first and foremost that the treatment is still experimental, and not yet widely available to cystic fibrosis patients. Furthermore, gene therapy will require that enough normal genes be added to correct a significant amount of defective cells. In addition, gene therapy will also need to be repeated every few months until it is possible to identify and correct "parent cells"  
10 which replace the defective cells. Improved delivery methods will also be necessary.

Yet other treatments are directed to symptoms which are a secondary result of the disease. A major problem of cystic fibrosis is secondary bacterial infections, which result in lung infections and inflammations. In fact, cystic fibrosis is lethal primarily due to chronic respiratory infections caused by several bacteria, culminating with  
15 chronic colonization with *Pseudomonas aeruginosa* (Goban, J.R. and Deretic, V. (1996) Microbiol Rev 60: 539-574). Treatment of such respiratory infections has been via antibiotics, but advances in this area have been few and far between. For example, the first new drug therapy developed exclusively for cystic fibrosis in 30 years was approved by the FDA in 1993, and was a mucous-thinning drug called Pulmozyme®.  
20 In 1997, the FDA approved another drug, TOBI® (tobramycin solution for inhalation), which is a reformulated version of a common antibiotic.

What is needed is an alternative to current methods of treating cystic fibrosis. Preferably, such methods will be effective in preventing bacterial infections and associated inflammations of the lungs and tracheal tissues.

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#### SUMMARY OF THE INVENTION

The present invention provides a method of treating a cell, comprising providing a cell comprising a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation, and exposing the cell to an alkalinizing agent.

30 In another aspect, the present invention provides a method of treating a cell, comprising providing a cell comprising a CFTR gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes, and exposing the cell to an alkalinizing agent.

In yet another aspect, the present invention provides a method of treating a cell, comprising providing a cell comprising a CFTR gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes, and exposing the cell to an alkalinizing agent, under conditions  
5 sufficient to decrease the hyperacidification. In one embodiment, the decrease is at least about 0.2 pH units. In another embodiment, the decrease is at least about 0.2 pH units but less than about 0.6 pH units. In yet another embodiment, the hyperacidification is decreased to levels observed in a cell in which the CFTR gene mutation has been corrected. In another embodiment, the cell is *in vivo*. In another embodiment, the cell is  
10 a lung epithelial cell. In another embodiment, the cell is a tracheal epithelial cell.

In yet another embodiment, the alkalinizing agent is a weak base; preferably, the weak base is selected from the group consisting of  $\text{NH}_4\text{Cl}$ , chloroquinone, and bicarbonate. In another embodiment, the alkalinizing agent is a pharmacological agent, wherein the pharmacological agent perturbs cellular function to inhibit or decrease acid  
15 production in the trans-golgi network or endosomes or both, or to promote base formation in the trans-golgi network or endosomes or both, such that the pH of the trans-golgi network or endosomes or both is increased; preferably, the pharmacological agent is selected from the group consisting of lansoprazole and omeprazol; most preferably, the pharmacological agent is selected from the group consisting of  
20 derivatives of lansoprazole and omeprazol, wherein the derivatives are effective to decrease the hyperacidification. In yet another embodiment, the alkalinizing agent is an exogenous factor introduced by a heterologous gene, wherein the factor acts to inhibit or decrease acid production in the trans-golgi network or endosomes or both, or to promote base formation in the trans-golgi network or endosomes or both, such that the pH of the  
25 trans-golgi network or endosomes or both is increased.

In yet another embodiment, the alkalinizing is added at a low concentration; preferably, the low concentrations range from about 0.01 to 0.1 mM.

In yet another embodiment, the alkalinizing agent is present in a form selected from the group consisting of a liquid, a vapor, a mist, a solid, and a powder.

30 The present invention further provides a method of treating a cell, comprising providing a cell comprising a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes, transforming the cell with a gene such that expression of the gene results in a decrease of the hyperacidification, and

growing the cell under conditions effective to express the gene. In one aspect, the gene encodes an influenzae M2 protein.

The present invention also provides a method of treating cystic fibrosis in a subject having cystic fibrosis, comprising administering a therapeutically effective amount of an  
5 alkalizing agent.

The present invention also provides a method of treating cystic fibrosis in a subject having cystic fibrosis, comprising administering an effective amount of an alkalizing agent under conditions effective to result in a decrease of hyperacidity of trans-golgi network or endosomes or both within cells expressing a CFTR gene mutation. In one  
10 embodiment, the alkalizing agent is administered to lung tissue of the subject. In one aspect, administering the alkalizing agent is effective to increase sialyltransferase in cells expressing a CFTR gene. In another aspect, administering the alkalizing agent is effective to decrease infection of lung tissue by pathogenic bacteria. In yet another aspect, administering the alkalizing agent is effective to decrease inflammation of the  
15 lung tissue. In yet another aspect, the alkalizing agent is present in a form selected from the group consisting of a liquid, a vapor, a mist, a solid, and a powder. In one embodiment, administering the alkalizing agent is intranasally. In another aspect, administering the alkalizing agent is through respiratory inhalation.

## 20 DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases as used herein are defined below:

The term "biocompatible" refers to compositions comprised of natural or synthetic materials, in any suitable combination, that remain substantially biologically unreactive  
25 in a subject or patient. The term "substantially unreactive" means that any response observed in a subject or patient is a subclinical response, *i.e.*, a response that does not rise to a level necessary for therapy.

The term "biologically active agent" and "therapeutic agent" refers to compositions that possess a biological activity or property having structural (for example, binding  
30 ability), regulatory, or biochemical functions. Moreover, as used herein, the term "agent" refers to biologically active agents and therapeutic agents, except where noted otherwise. Biological activities include activities associated with biological reactions or events in a subject or patient that allow the treating, detection, monitoring, or characterization of biological reactions or events. Biological activities include, but are

not limited to, therapeutic activities (for example, the ability to improve biological health or prevent the continued degeneration associated with an undesired biological condition), targeting activities (for example, the ability to bind or associate with a biological molecule or complex), monitoring activities (for example, the ability to  
5 monitor the progress of a biological event or to monitor changes in a biological composition), imaging activities (for example, the ability to observe or otherwise detect biological compositions or reactions), and signature identifying activities (for example, the ability to recognize certain cellular compositions or conditions and produce a detectable response indicative of the presence of the composition or condition). The  
10 agents of the present invention are not limited to these particular illustrative examples. Indeed any biologically active agent or therapeutic agent may be used including compositions that deliver or destroy biological materials, cosmetic agents, and the like. The agents may comprise, for example, nucleic acids, antibiotics, chemotherapeutic agents, proteins, and organic or inorganic molecules or compounds. Such agents may or  
15 may not further comprise common pharmaceutically acceptable compositions (for example, adjuvants, excipients, or diluents).

The term "agonist" refers to a molecule which, when interacting with a biologically active molecule, causes a change (for example, enhancement) in the biologically active molecule, which modulates the activity of the biologically active molecule. Agonists  
20 include proteins, nucleic acids, carbohydrates, or any other molecules that bind or interact with biologically active molecules. For example, agonists can alter the activity of gene transcription by interacting with RNA polymerase directly or through a transcription factor.

The terms "antagonist" or "inhibitor" refer to a molecule which, when interacting  
25 with a biologically active molecule, blocks or decreases the biological activity of the biologically active molecule. Antagonists and inhibitors include proteins, nucleic acids, carbohydrates, or any other molecules that bind or interact with biologically active molecules. Inhibitors and antagonists can effect the biology of entire cells, organs, or organisms (for example, an inhibitor that slows tumor growth).

30 The term "alkalinizing agent" refers to a compound, regardless of size, biological activity, mode of action, and so forth, which, upon contacting a cell, effects a reduction in the pH of at least the TGN or the endosomes, or preferably of both, of a CF cell. Thus, an alkalinizing agent can be any reagent which can reduce the pH of the TGN or of the endosomes or preferably of both; preferably, the pH should be reduced to about

the pH observed in a non-CF cell, or in a corrected CF cell. An alkalinizing agent is preferably one of a few types. One type comprises basic compounds which act directly by reducing the pH chemically. Another type comprises pharmacological agents which perturb or modulate cellular function to inhibit or decrease acid production in the TGN  
5 or the endosomes or both, or to promote base formation in the TGN or endosomes or both, such that the pH is increased. Yet another type is an exogenous factor introduced by a heterologous gene, which acts to inhibit or decrease acid production in the TGN or endosomes or both, or to promote base formation in the TGN or endosomes or both, such that the pH is increased.

10 The term "weak base" refers to the state of association and dissociation of protons with the base, such that at physiological pH the base is capable of existing in both states in an equilibrium; the dissociated form is capable of moving slowly from an external environment into a subcellular compartment, passing the subcellular membrane in an uncharged form, and in an acidic subcellular compartment is capable of being  
15 protonated where it is essentially trapped. Preferably, a weak base is a compound which will accumulate in acidic cellular compartments and has the capacity to normalize the pH of the compartment by neutralizing an acidic pH to a more physiological pH. Examples of organic bases include but are not limited to primary, secondary, and tertiary amines (such as ammonia), chloroquine, and bicarbonate

20 The term "pharmacological agent" refers to agents which perturb or modulate cellular function to inhibit or decrease acid production in the TGN or endosomes or both, or to promote base formation in the TGN or endosomes or both, such that pH is increased. For example, in one aspect, such agents target  $\text{Na}^+$  channels, proton ATPases, or  $\text{Na}^+/\text{K}^+$  ATPases. In one embodiment, pharmacological agents which  
25 affect  $\text{Na}^+$  channels inhibit  $\text{Na}^+$  conductance, preferably resulting in an open channel such that a  $\text{Na}^+$  efflux from the TGN occurs, resulting in an increase in the pH. One example of such an agent is amiloride. In another embodiment, pharmacological agents inhibit proton ATPases; such agents include but are not limited to bafilocin A, omeprazole and anti-acids, including but not limited to lansoprazole and omeprazole.  
30 Such agents may be chemically modified, in order to modify toxicity and in order to modify activity relative to inhibiting proton ATPases. In another embodiment, pharmacological agents modulate  $\text{Na}^+/\text{K}^+$  ATPases. Such agents enhance  $\text{Na}^+$  accumulation in the lumen of TGN, by activating  $\text{Na}^+/\text{K}^+$  ATPase, and thereby reversing acidification of the TGN

The term "change" or "modulate" refers to a change in the biological activity of a biologically active molecule. Modulation can be an increase or a decrease in activity, a change in binding characteristics, or any other change in the biological, functional, or immunological properties of biologically active molecules.

5       The term "therapeutically effective amount" is a functional term referring to an amount of material needed to make a qualitative or quantitative change in a clinically measured parameter for a particular subject. For example, prior to administration, the subject may exhibit measurable symptoms of disease (for example, viral antigen load, clotting time, serum analyte level, vitamin or nutrient deficiency, etc), which upon  
10 administration of a therapeutically effective amount the measurable symptom is found to have changed. A therapeutically relevant effect relieves to some extent one or more symptoms of a disease or condition or returns to normal either partially or completely one or more physiological or biochemical parameters associated with or causative of the disease.

15       The terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a subject or patient, preferably a mammal, most preferably a human, and that the materials do not substantially produce, for example, adverse or  
20 allergic reactions when administered to a subject or patient, or can be administered without the production of undesirable physiological effects such as nausea, dizziness, gastric upset, toxicity and the like.

The term "aqueous component" refers to the component of a composition that contains water (or is soluble in water). Where water is used, it may or may not contain  
25 salt(s) and may or may not be buffered. Thus, a variety of such components are contemplated including, but not limited to, distilled water, deionized water, normal saline, and phosphate buffered saline.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which a compound is administered. Such pharmaceutical carriers can be sterile liquids, such as  
30 water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.



The term "intranasally" refers to the introduction of a pharmaceutical composition within the nasal cavity.

The term "respiratory inhalation" refers to the introduction of a pharmaceutical composition within the respiratory tract.

5 The term "subject" refers to any animal (for example, warm blooded mammal), including, but not limited to, humans, non-human primates, rodents, farm animals (for example, cattle, horses, pigs, goats, and sheep) and the like, that is to be the recipient of a particular treatment. The terms "subject" and "patient" are used interchangeably.

The term "gene" refers to a nucleic acid (for example, DNA) sequence that  
10 comprises coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (for example, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene  
15 and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences that are located 3' or downstream of the coding region and  
20 which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may  
25 contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include  
30 sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3'

flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "heterologous" when used in reference to a gene refers to a gene encoding a factor that is not in its natural environment (in other words, has been altered by the hand of man). For example, a heterologous gene includes a gene from one species introduced into another species. A heterologous gene also includes a gene native to an organism that has been altered in some way (for example, mutated, added in multiple copies, linked to a non-native promoter or enhancer sequence, etc.). An "exogenous factor" is a factor which does not naturally occur in a cell.

The terms "mutation" or "mutant" or "modified" when made in reference to a gene or to a gene product refer, respectively, to a gene or to a gene product which displays modifications in sequence and/or functional properties (in other words, altered characteristics) when compared to the wild-type gene or gene product. The terms "variant" and "mutant" when used in reference to a polypeptide refer to an amino acid sequence that differs by one or more amino acids from another, usually related or wild-type, polypeptide.

The terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

The term "antisense" is used in reference to DNA or RNA sequences that are complementary to a specific DNA or RNA sequence (for example, mRNA). Included within this definition are antisense RNA ("asRNA") molecules involved in gene regulation by bacteria. Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced into an embryo, this transcribed strand combines with natural mRNA produced by the embryo to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (in other words, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (in other words, "positive") strand.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

5 The term "transgene" refers to a foreign gene that is placed into an organism. The term "foreign gene" refers to any nucleic acid (for example, gene sequence) that is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not reside in the same location as does the naturally-occurring gene.

10 The term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

The term "expression vector" refers to a recombinant DNA molecule containing a  
15 desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination  
20 and polyadenylation signals.

The terms "overexpression" and "overexpressing" and grammatical equivalents, are used in reference to levels of mRNA to indicate a level of expression approximately 3-  
fold higher than that typically observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to  
25 those skilled in the art including, but not limited to Northern blot analysis. Appropriate controls are included on the Northern blot to control for differences in the amount of RNA loaded from each tissue analyzed (for example, the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of normalizing or standardizing the RAD50  
30 mRNA-specific signal observed on Northern blots).

The term "gene transfer system" refers to any means of delivering a composition comprising a nucleic acid sequence to a cell or tissue. For example, gene transfer systems include, but are not limited to vectors (for example, retroviral, adenoviral, adeno-associated viral, and other nucleic acid-based delivery systems), microinjection of

naked nucleic acid, dendrimers, and polymer-based delivery systems (for example, liposome-based and metallic particle-based systems). As used herein, the term "viral gene transfer system" refers to gene transfer systems comprising viral elements (for example, intact viruses and modified viruses) to facilitate delivery of the sample to a  
5 desired cell or tissue.

The term "transfection" refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome  
10 fusion, lipofection, protoplast fusion, retroviral infection, biolistics, and dendrimers.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

15 The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the  
20 chromosomes. The term "transient transfectant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques.

25 The terms "contacted" and "exposed," when applied to target cells or tissues, are used to describe the process by which a composition (for example, comprising an alkalinizing agent) is delivered to a target cell or tissue are placed in contact (for example, direct contact) with the target cell or tissue.

The term "cell culture" refers to any *in vitro* culture of cells. Included within this  
30 term are continuous cell lines (for example, with an immortal phenotype), primary cell cultures, finite cell lines (for example, non-transformed cells), and any other cell population maintained *in vitro*.

The term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments can consist of, but are

not limited to, test tubes and cell culture. The term "*in vivo*" refers to the natural environment (for example, an animal or a cell) and to processes or reaction that occur within a natural environment.

The term "cystic fibrosis transmembrane conductance regulator" or "CFTR" refers to a protein which functions as an apical membrane chloride channel; a mutation in the gene *CFTR* which encodes CFTR results in cystic fibrosis (Schwiebert E. M., *et al.* (1999) *Physiol Rev* 79, S145-66; Bradbury, N.A. (1999) *Physiol. Rev* 79: S175-91). Various CFTR mutations in cystic fibrosis affect the processing, intracellular localization, and function of this protein (Schwiebert E. M., *et al.* (1999) *Physiol Rev* 79, S145-66; Bradbury, N.A. (1999) *Physiol. Rev* 79: S175-91), as well as affect the function of other ion transporters. The result of these mutations is the production in the body of an abnormally thick, sticky mucous, due to the faulty transport of sodium and chloride within the cells lining organs, such as the lungs and pancreas.

The terms "a cell having CF" or "a CF cell" refer to a cell which possesses a mutation in a *CFTR* gene which results in cystic fibrosis. The term "a non-CF cell" or grammatical equivalents refer to a cell which does not possess a mutation in the *CFTR* gene which results in cystic fibrosis. The term "a corrected CF cell" or grammatical equivalents refer to a cell which possess a mutation in a *CFTR* gene which results in cystic fibrosis, but in which the defect has been corrected; typically, such correction is by transformation of the cell with a normal *CFTR* gene, but other corrections are also contemplated.

The term "trans-golgi network" or "TGN" refer to a portion of the Golgi apparatus. The Golgi apparatus in eukaryotic cells receives newly synthesized proteins and lipids from the endoplasmic reticulum and delivers them to their correct destination in the cell. These molecules pass, in sequence, through an ordered array of Golgi compartments each capable of making a specific, yet different, set of covalent modification to the lipids and proteins that pass through. Macromolecules entering the Golgi apparatus share a common pathway up to the trans-golgi network (TGN) where they are separated from each other in preparation for final delivery. In animal cells, the Golgi apparatus comprises a single, compact reticulum near to one side of the nucleus; in cross section, a central feature is a stack of closely apposed and flattened cisternae. The TGN lies on the trans side of the Golgi, furthest away from the endoplasmic reticulum. Late events in modification of proteins and lipids typically occur in the TGN. For example, completion of protein complex oligosaccharides and sulphation occur in the TGN.

The term "endosomes" refers to an organelles of the endocytic pathway intermediate between the plasma membrane and lysosomes. Endosomes are the first compartment to which ligands, membrane components and fluid internalized by fluid phase and receptor-mediated endocytosis are delivered following endocytosis from the cell surface. Endosomes perform a sorting function, returning the bulk of the membrane to the cell surface while retaining specific components for transcytosis or transport to lysosomes. Endosomes are morphologically complex, comprising a system of interconnected membrane bounded tubules (approximately 50 nm in diameter), cisternae, and vesicles. Membrane flow through the system is considerable with the result that the organelles are highly plastic and may form an extended reticulum with elements continually undergoing fusion and fission.

The term "hyperacidity" refers to a decrease in pH, compared to a control. For example, with respect to a measured pH of the TGN or endosomes or both in a CF cell, hyperacidity means that the pH in these organelles in the CF cell is at least about 0.2 pH units less than would be observed in a non-CF cell or in a corrected CF cell. A "decrease" or "reduction" in hyperacidity in the pH of the TGN or endosomes or both in a CF cell need be complete; in other words, it need not match the levels observed in a non-CF cell or corrected CF cell. The present invention contemplates a partial reduction or partial decrease in hyperacidification. While not limited to any particular measurement, a "decrease" can be at least about 10%, or more preferably greater than at least about 20%, or still more preferably at least about 50% of the increase of acidity of the TGN or endosomes or both of a CF cell with respect to those of a control non-CF or CF-corrected cell.

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (for example, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

The terms "purify" or "to purify" refer to the removal of contaminants from a sample. The term "purified" refers to molecules, such as nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated. An

"isolated nucleic acid sequence" is therefore a purified nucleic acid sequence.

"Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated. As used herein, the term "purified" or "to purify" also refers to the  
5 removal of contaminants from a sample. The removal of contaminating proteins results in an increase in the percent of polypeptide of interest in the sample. In another example, recombinant polypeptides are expressed in plant, bacterial, yeast, or mammalian host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

10 The term "medical devices" includes any material or device that is used on, in, or through a patient's body in the course of medical treatment (for example, for a disease or injury). Medical devices include, but are not limited to, such items as nebulizers, small particle aerosol generators, inhalers with a propellant and the like.

As used herein, the term "sample" is used in its broadest sense. In one sense it can  
15 refer to a biopolymeric material. In another sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be animal, including, human, fluid (for example, blood, plasma and serum), solid (for example, stool), tissue, liquid foods (for example, milk), and solid foods (for example, vegetables) Biological samples include blood products, such as  
20 plasma, serum and the like. Environmental samples include environmental material such as surface matter, soil, water, crystals, and industrial samples. These examples are not to be construed as limiting the sample types applicable to the present invention.

#### GENERAL DESCRIPTION OF THE INVENTION

25 The present invention relates to methods of treating cystic fibrosis, and in particular to exposing cystic fibrosis cells to alkalinizing agents.

The main cause of high morbidity and mortality in cystic fibrosis (CF) are chronic respiratory infections and associated inflammations (Welsh, M.M *et al.* (1995) in The metabolic and molecular basis of inherited disease, eds. Scriver, C.R. Beaudet, A.L. Sly,  
30 W.S. and Valle, D. (McGraw-Hill, Inc., New York), Vol. III, pp 3799-3876). A definitive link still remains to be established between the primary defect in the *CTFR* gene, which encodes the cystic fibrosis transmembrane conductance regulator (CFTR), and the predilection for infections and excessive inflammation in CF.

It is widely recognized that progressive deterioration of respiratory function in CF is the result of persistent bacterial colonization, culminating with chronic colonization with *Pseudomonas aeruginosa* (Govan, J.R. and Deretic, V. (1996) *Microbiol Rev* 60: 539-570). Furthermore, anti-inflammatory therapy is beneficial and slows down  
5 deterioration of lung disease in CF (Konstan, M.W. *et al.* (1995) *N Engl J Med* 332: 848-854). Significantly elevated levels of TNF- $\alpha$ , IL-1 and IL-8 and reduced amounts of the major anti-inflammatory cytokine IL-10 in bronchoalveolar lavage fluid are typical of CF (Bonfield, T.L. *et al.* (1995) *Am J Respir Crit Care Med* 152: 2111-2118).  
10 In addition to the inflammation provoked by infections, it appears that some level of spontaneous inflammation in CF may exist possibly even before colonization with infectious agents (Khan, T.Z. *et al.* (1995) *Am J Respir Crit Care Med* 151: 1075-1082).

Some of the hyper-excitability of CF tissues has been attributed to activation of NF- $\kappa$ B (a transcriptional factor necessary for expression of many pro-inflammatory cytokines) (DiMango, E. *et al.* (1989) *J Clin Invest* 101: 2598-2605), as exogenous  
15 stimuli (for example TNF- $\alpha$ , IL-1, exposure to *P. aeruginosa* or its products (DiMango, E. *et al.* (1989) *J Clin Invest* 101: 2598-2605; DiMango E. *et al.* (1995) *J Clin Invest* 96: 2204-2210)) appear to activate NF- $\kappa$ B in CF epithelial cells to higher levels than in normal controls. However, the reported elevated basal levels of NF- $\kappa$ B activation in CF are highly dependent on growth conditions, in other words, the presence of serum,  
20 which increases the basal levels of IL-8 and NF- $\kappa$ B. Furthermore, RSV-stimulation elicits similar IL-8 levels in CF and adenovirus CFTR-corrected cells (Black, H.R. *et al.* (1998) *Am J Respir Cell Mol Biol* 19: 210-215).

Whether or not the imbalance between pro- and anti-inflammatory cytokines in CF is due to intrinsic properties directly linked to CFTR mutations, the binding of *P.*  
25 *aeruginosa* to CF epithelial cells, which is enhanced due to under-sialylation of glycolipids in CF (Imundo, L. *et al.* (1995) *Proc Natl Acad Sci USA* 92: 3019-3023), has been shown to activate NF- $\kappa$ B (DiMango, E. *et al.* (1998) *J Clin Invest* 101: 2598-2605; Davies, J. *et al.* (1999) *Eur Respir J* 13: 565-570) and IL-8 production. Moreover, CF cells may have prolonged inflammatory response upon exposure to *P.*  
30 *aeruginosa*.

However, the exact connection between CFTR mutations, their physiological effects, and *P. aeruginosa* infections and associated inflammations has not been fully established, as is reflected in the multitude of proposals explaining the predilection to bacterial infection in CF (Barasch, J. *et al.* (1991) *Nature* 52: 70-73; Smith, J.J. *et al.*



(1996) Cell 85: 229-236; Goldman, M.J. *et al.* (1997) Cell 88: 53-560; Knowles, M.R. *et al.* (1997) J Clin Invest 100: 2588-2595; Matsui, H. *et al.* (1998) Cell 95: 1005-1015; Levison, H. *et al.* (1983) Eur J Respir Dis Suppl 127: 102-117; Guggino, W.B. (1999) Cell 96: 607-610; Kelley, T.J. and Drumm, M.L. (1998) J Clin Invest 102: 1200-1207; Yu, H. *et al.* (2000) Infect Immun 68: 2142-2147; Pier, G.B. *et al.* (1996) Science 271: 64-67; Pier, G.B. *et al.* (1997) Proc Natl Acad Sci USA 94: 12088-12093; Pier, G.B. (2000) Proc Natl Acad Sci USA 97: 8822-8828; and Imundo, L. *et al.* (1995) Proc Natl Acad Sci USA 92: 3019-3023). These proposals are summarized as follows.

The first proposal is that destruction of *P. aeruginosa* by airway epithelium-derived antibiotic peptides (defensins) in secretions is impaired by the abnormally high salt concentrations. It has been reported that defensins are impaired by high salt in CF lung secretions (Smith, J.J. *et al.* (1996) Cell 85: 229-236; Goldman, M.J. *et al.* (1997) Cell 88: 53-560). However, according to several studies, the electrolyte composition of the airway surface fluid does not appear to differ between normal and CF respiratory epithelia (Knowles, M.R. *et al.* (1997) J Clin Invest 100: 2588-2595). A second proposal is based upon the concept of low volume and impaired mucociliary clearance. In contrast to the high salt hypothesis, the low volume hypothesis proposes that uninhibited amiloride-sensitive epithelial sodium channel (ENaC) leads to hyperabsorption of sodium and water, leading to low airway surface liquid volume, which in turn impedes mucociliary clearance (Matsui, H. *et al.* (1998) Cell 95: 1005-1015). However, mucociliary clearance alone cannot explain the predilection for *P. aeruginosa*, as there are substantial differences between CF and diseases such as primary ciliary dyskinesia (Levison, H. *et al.* (1983) Eur J Respir Dis Suppl 127: 102-117), and primary ciliary dyskinesia also results in impaired mucociliary clearance, but does not result in *P. aeruginosa* infection. Thus, the high salt and low volume proposals remain controversial (Guggino, W.B. (1999) Cell 96: 607-610).

Yet another hypothesis, based upon a different approach, is that reduced inducible nitric oxide synthase (iNOS) production and NO output play a role in susceptibility to *P. aeruginosa* (Kelley, T.J. and Drumm, M.L. (1998) J Clin Invest 102: 1200-1207). However, infection models in iNOS transgenic mice support only a limited role for NO in innate defenses against *P. aeruginosa* (Yu, H. *et al.* (2000) Infect Immun 68: 2142-2147).

Recently, yet another hypothesis has been proposed that CFTR itself acts as a receptor for *P. aeruginosa* in bacterial uptake by the epithelial cells (Pier, G.B. *et al.*

(1996) Science 271: 64-67; Pier, G.B. *et al.* (1997) Proc Natl Acad Sci USA 94: 12088-12093; Pier, G.B. (2000) Proc Natl Acad Sci USA 97: 8822-8828). In this model, *P. aeruginosa* is envisioned as normally taken up by epithelial cells and eliminated from the respiratory tract by desquamation (G36). It has been proposed that  
5 CFTR itself acts as a receptor for *P. aeruginosa* in bacterial uptake by the epithelial cells (G36). In CF, the mutant deltaF508 CFTR, which is not properly folded and remains trapped in the ER, does not traffic to the plasma membrane. This in turn would eliminate the proposed receptor for *P. aeruginosa* uptake by the epithelial cells. If it is assumed that epithelial uptake of the bacterium could be a part of the clearance process,  
10 the lack of CFTR would then translate into less efficient elimination of *P. aeruginosa*. However, a role for bacterial uptake by respiratory epithelial cells remains to be investigated and has not been established.

Yet another proposal is based upon the concept that epithelial glycoconjugates are under-sialylated, resulting in increased adherence of *P. aeruginosa* to CF respiratory  
15 epithelium. It has been demonstrated that glycoproteins and glycolipids on the plasma membrane of CF cells display altered sialylation, and that under-sialylated glycoconjugates act as adhesion receptors for bacterial pathogens in CF (Imundo, L. *et al.* (1995) Proc Natl Acad Sci USA 92: 3019-3023; and Saiman, L. and Prince, A. (1993) J Clin Invest 92: 1875-1880). Since adhesion of a pathogen to host tissues  
20 occurs at the very initial stages of infection, the increased bacterial association with CF respiratory epithelial cells most likely represents a critical point in the colonization of the respiratory tract in CF. As the major CF pathogens *P. aeruginosa* and *Staphylococcus aureus* preferentially adhere to unsialylated glycoconjugates such as aGM1 (Drivan, H.C. *et al.* (1988) Proc Natl Acad Sci USA 85: 6157-6161), it is  
25 possible that the under-sialylation of surface molecules on CF epithelial cells promotes bacterial colonization (Imundo, L. *et al.* (1995) Proc Natl Acad Sci USA 92: 3019-3023; and Saiman, L. and Prince, A. (1993) J Clin Invest 92: 1875-1880).

However, the connection between CFTR defect and altered sialylation remains elusive (Dosanjh, A. *et al.* (1994) Am J Physiol 266: C360-C3666). In one model, it has  
30 been proposed that CFTR plays a role in facilitating acidification of intracellular compartments, such as the trans-Golgi network (TGN), by allowing entry of anions (Cl<sup>-</sup>) and thus maintaining charge neutrality as protons are pumped into the lumen of these organelles (Barasch, J. *et al.* (1991) Nature 352: 70-73). According to this proposal, a loss of CFTR and chloride conductance would result in an increased pH of TGN

(Barasch, J. *et al.* (1991) *Nature* 352: 70-73; Barasch, J. and al-Awqati, Q. (1993) *J Cell Sci* 17: 229-233). This hypothesis provided an attractive explanation for reduced sialylation of glycoconjugates, as sialyltransferase would act under suboptimal pH conditions in the TGN of CF cells. However, despite the prediction that TGN would be alkaline, a subsequent study failed to detect alkalization of TGN in CF (Seksek, O. *et al.* (1996) *J Biol Chem* 271: 15542-15548).

The methods of the present invention are based upon the unexpected and surprising discovery that the trans-Golgi network (TGN) and cellubrevin-containing endosomes are hyperacidified in the presence of mutant CFTR in CF lung epithelial cells, and that correction of hyperacidification can reverse the effects of such hyperacidification. Such effects include increased infections, resulting from under-sialylation with a concomitant increase in adhesion of pathogenic bacteria and from decreased or impaired membrane flow and endocytosis with a concomitant decrease of bacterial elimination, as well as increased inflammations. These findings are in direct contrast with previous reports that the pH of intracellular organelles may be elevated in CF or that there are no differences between normal and CF cells.

This discovery resulted from the application of pH sensitive, ratiometric GFP technology (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195) and targeting of the pH sensitive GFP to TGN and to endosomes in live cells, which permitted the detection of hyperacidification of these compartments in CF cells; these results, which are described in greater detail in the following Examples, are summarized as follows.

A newly developed pH sensitive GFP tool (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195), referred to as pHluorin GFP, is a ratiometric fluorescence tool for in vivo pH determination: its absorption at 410 and 470 nm is pH sensitive, and therefore it can be used to determine pH by exciting the fluorophore at 410 and 470 nm and then measuring the ratio of fluorescence emission at 510 nm. This new tool was used to examine the question of TGN and endosomal acidification in CF cells.

Three fusion constructs were used; in each case, pHluorin GFP was fused to a protein which targets a specific subcellular compartment. These three probes were cellubrevin-pHluorin GFP, TGN38-pHluorin GFP and glycosylphosphatidylinositol (GPI)-pHluorin GFP (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195), which target cellubrevin-containing endosomes, the TGN, and the plasma membrane, respectively. The first two constructs have lumenally exposed pH sensitive GFP, whereas the third construct localizes to the plasma membrane where it is exposed to the extracellular

fluid. The endosomal-, TGN- and plasma membrane-targeted pHluorin GFP probes were transfected into well characterized cell lines, IB3-1, C-38 and S-9 (Zeitlin, P.L. *et al.* (1991) *Am J Respir Cell Mol Biol* 4: 313-319; and Egan, M. *et al.* (1992) *Nature* 358: 581-584).

5 Cell line IB3-1 is a human bronchial epithelial cell line derived from a CF patient with a DF508/W1282X CFTR mutant genotype (Zeitlin, P.L. *et al.* (1991) *Am J Respir Cell Mol Biol* 4: 313-319). Cell lines C-38 and S-9 are stably transfected derivatives of IB3-1 cells corrected for chloride conductance by introduction of a functional CFTR (Egan, M. *et al.* (1992) *Nature* 358: 581-584).

10 The ratio of emission at 508 nm upon excitation at 410 nm vs. 470 nm was recorded using filter sets as previously described (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195). The ratio of emission was determined using Merlin program (LSR, Olympus, Melville, NY, USA). The fluorescence of GPI-pHluorin GFP at pH 7.4 and pH 5.5 was first determined. A standard curve was generated within the working range of pHluorin  
15 GFP (pH 7.4 - 5.5), and ratio of emission was plotted as a function of pH (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195).

C-38 and S-9 cells all showed identical fluorescence dependence on the pH of the external buffer, as determined by measuring the fluorescence of GPI-pHluorin GFP. In addition, at the end of each experiment with all GFP fusions, the pH-gradient was  
20 collapsed by incubating cells in 10 mM monensin and 10 mM nigericin for 30 min at 37°C in buffer A (25 mM HEPES, 119 mM NaCl, 2.5 mM KCl, 2mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub>, 30 mM Glucose), at pH 7.4 or pH 5.5, and the ratios were recorded for internal standards.

The pH of TGN was probed with TGN38-pHluorin GFP (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195). The pH of TGN compartment in C-38 cells was pH  
25  $6.6 \pm 0.1$  and in S-9 cells  $6.7 \pm 0.1$ , whereas the corresponding pH in IB3-1 CFTR mutant cells was pH  $6.0 \pm 0.1$  (Table 1). Thus, the luminal TGN pH-values in IB3-1 cells were 0.7 and 0.6 pH-units lower than in C-38 and S-9 cells, respectively ( $P=0.0001$ ), showing that this compartment is hyperacidified in CFTR mutant cells.

30 Next, the pH of cellubrevin-containing endosomal compartments was probed with pHluorin GFP fused to cellubrevin (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195). The pH of cellubrevin-containing endosomes was  $6.7 \pm 0.1$  for both CFTR corrected cells, C-38 and S-9, compared to pH  $6.2 \pm 0.1$  in CFTR mutant cells (Table 1).

To demonstrate that the differences in pH were due to organelle acidification, the pH-gradient was collapsed with monensin and nigericin. Upon pH-gradient collapse the endosomal pH changed to 7.2, approaching the pH of the incubation buffer. Thus, cellubrevin-labeled compartments in CF mutant cells show hyperacidification of a 0.5  
5 pH unit ( $P=0.0001$ ).

These results were confirmed using another set of a well characterized CF cell line and its stably transfected derivatives. The cell line is CFT1 (Olsen, J.C. *et al.* (1992) *Hum Gene Ther* 3: 253-266), which is derived from the tracheal epithelium of a CF patient homozygous for the CFTR<sup>D</sup>F508 mutation; its stably transfected derivatives  
10 included CFT1-LCFSN, expressing the wild-type CFTR gene, CFT1-<sup>D</sup>F508, transfected with <sup>D</sup>F508 mutant CFTR gene, and CFT1-LC3, the vector transfected control cell line. Repeated measurements of defined regions of interest (ROI) colocalizing with endocytic tracers or sialyltransferase did not differ from the pH values of randomly chosen ROI that served to generate the results shown in Table 1.

15 The observation of hyperacidification of the TGN and endosomes is in contrast with previous models, where alkalinization of this normally mildly acidified organelle was predicted in CF on the basis of CFTR-dependent chloride conductance (Barasch, J. *et al.* (1991) *Nature* 352: 70-73; and Barasch, J. and al-Awqati, Q. (1993) *J Cell Sci* 17: 229-233). It is important to note that these results are not easily explained by the proposed  
20 action of CFTR as a chloride channel, with chloride ions acting to maintain electroneutrality by compensating for the positive luminal charge generated by protons pumped by the H<sup>+</sup>-ATPase (Barasch, J. *et al.* (1991) *Nature* 352: 70-73).

Although it is not necessary to understand the mechanism in order to use the present invention, and it is not intended that the present invention be so limited, it is  
25 contemplated that the regulatory functions of CFTR, such as the CFTR-dependent inhibition of the epithelial sodium channel (ENaC) in human respiratory epithelial cells (Bradbury, N. A. (1999) *Physiol Rev* 79, S175-91; Stutts, M. J. *et al.* (1995) *Science* 269, 847-850; Ismailov, II *et al.* (1996) *J Biol Chem* 271: 4725-32; Stutts, M. J *et al.* (1997) *J Biol Chem* 272: 14037-14040; Kunzelmann, K. and Schreiber, R. (1999) *J.*  
30 *Membrane Biol* 168; 1-8; Reddy, M. M. et al (1999) *Nature* 402: 301-304), may play a role in hyperacidification, rather than its proposed function as a chloride channel. Under this hypothesis, excess positive charge, caused by accumulation of H<sup>+</sup> in the lumen of this organelle, may be compensated by Na<sup>+</sup> efflux into the cytosol, thus dissipating the electrogenic charge differential (Zen, K. *et al.* (1992) *J Cell Biol* 119: 99-110) and

allowing the development of a greater transmembrane pH gradient. The proton pump is exquisitely sensitive to membrane potential build up. In normal cells, an inactive ENaC and an active  $\text{Na}^+/\text{K}^+$ -ATPase would maintain luminal positive charge and thus counteract acidification. In CF cells, in the absence of CFTR-dependent inhibition of ENaC transport (Bradbury, N. A. (1999) *Physiol Rev* 79: S175-91; Stutts, M. J. *et al.* (1995) *Science* 269: 847-850; Schreiber, R. *et al.* (1999) *Proc Natl Acad Sci USA* 96: 5310-5315; Ismailov, II. *et al.* (1996) *J Biol Chem* 271: 4725-4732; Stutts, M. J. *et al.* (1997) *J Biol Chem* 272: 14037-14040; and Kunzelmann, K. and Schreiber, R. (1999) *J. Membrane Biol* 168: 1-8), the sodium channel is open and  $\text{Na}^+$ -efflux can compensate for the  $\text{H}^+$ -associated positive charge build up, thus neutralizing the membrane potential and facilitating  $\text{H}^+$ -ATPase action which results in TGN acidification.

Another aspect of the present invention is the discovery that  $\text{H}^+$ -ATPase appears to be involved in the hyperacidification of the TGN and endosomes, and that ENaC and sodium also appear to be involved.

Thus, the model proposed above was tested by first examining the role of  $\text{H}^+$ -ATPase in the acidification of the TGN and endosomes. In the test, 100 nM bafilomycin A (added for 2.5 h at  $37^\circ\text{C}$  to cellubrevin-pHluorin GFP or TGN38-pHluorin transfected IB3-1, C-38 and S-9 cells) was able to inhibit acidification of cellubrevin-labeled endosomes and TGN.

Next, the role of ENaC in acidification was tested. First, the addition of 100 mM amiloride (in buffer A to cellubrevin-pHluorin transfected IB3-1 cells) led to an increase in pH of 1 unit after 2 hours of incubation. Next, incubation of transfected IB-31 cells in the absence of  $\text{Na}^+$  normalized the pH of both compartments, which supports the role of  $\text{Na}^+$  conductance in hyperacidification of intracellular organelles in CF. Subsequent reintroduction of  $\text{Na}^+$  reversed the pH to abnormally high values.

Thus, incubation of cellubrevin-pHluorin GFP or TGN38-pHluorin GFP transfected cells, in  $\text{Na}^+$ -free buffers (buffer A: 119 mM LiCl instead of NaCl at pH 7.4) for 30 min at  $37^\circ\text{C}$  normalized the pH of TGN (pH  $6.9 \pm 0.2$ ,  $P=0.002$ ) and cellubrevin-labeled compartments (pH  $6.7 \pm 0.2$ ,  $P=0.0113$ ) in IB3-1 cells. Sodium was re-introduced by replacing LiCl solution with  $\text{Na}^+$ -based buffer A for 60 min, reversing the pH to abnormally acidic values for TGN (pH  $6.0 \pm 0.2$ ,  $P=0.038$ ) and cellubrevin-labeled endosomes (pH  $5.9 \pm 0.2$ ,  $P=0.0004$ ). The observed differences in acidification patterns

between cellubrevin-labeled endosomes and TGN in the ion substitution experiments most likely reflect contribution of additional ion pumps and channels.

Acidification of TGN and endosomes in CF cells is contemplated to have far-reaching physiological consequences. A hyperacidified TGN can lead to altered protein and lipid glycosylation manifested as decreased sialylation, since sialyltransferases responsible for protein modification have bell-shaped pH-optima (Busam, K. and Decker, K. (1986) *Eur J Biochem* 160: 23-30); this would be analogous to results expected from the alkalization hypothesis (Barasch, J. *et al.* (1991) *Nature* 352: 70-3),

Indeed, another aspect of the discovery of the present invention is that sialyltransferases are localized in the same cellular compartments which were hyperacidified, which could explain under-sialylation of glycoconjugates in CF cells. Thus, fluorescently-labeled peanut agglutinin, which recognizes unsialylated galactosyl (b-1,3) N-acetylgalactosamine (Poschet, J. F. *et al.* (2000) *Methods in Enzymol.*), binds better to CF cells (IB3-1, CFT1, CFT1-D508, and CFT1-LC3) than to non-CF cells to the (CFTR corrected C-38, S-9 or CFT1-LCFSN cells) in a process which is neuraminidase sensitive. Furthermore, sialyltransferases co-localized with the TGN-specific GFP, as observed in co-transfections of TGN38-pHluorin and myc-tagged a-2,6-sialyltransferase Sttyr isoform constructs in IB3-1, C-38 and S-9. These observations indicate that the pH was measured in a compartment relevant for sialyltransferase activity, and thus may explain under-sialylation of glycoconjugates in CF cells. Alterations in glycoconjugates are contemplated to lead to altered bacterial adhesion and downstream pro-inflammatory signaling in CF.

Yet another aspect of the discovery of the present invention is that treatment of CF cells with an alkalizing agent corrected the pH of the TGN and endosomes to match that of corrected CF cell lines. In one embodiment, the cells were treated with a weak base at a low concentration. Treatment of CF cells with an alkalizing agent also restored normal patterns of PNA binding to CF cells. Thus, pH correction of TGN in CF cells can lead to a restoration of normal sialylation.

These observations, from the treatment of CF cells with a weak base to neutralize pH in acidic intracellular compartments, indicate that hyperacidification is responsible for under-sialylation of glycoconjugates in CF. Treatment of CF cells with a low concentration of ammonium chloride decreased binding of PNA to the CF cells, presumably by normalizing sialylation in CFTR mutant cells and reversing this CF defect. Reported attempts to address the problem of altered pH in CF using ammonia

(Jiang, X. *et al.* (1997) *Am J Physiol* 273: L913-20) describe the addition of high concentrations (10  $\mu$ M) of ammonium chloride, which did not correct the defect but instead decreased sialylation and increased PNA binding to both normal and CF cells. These reports are nevertheless consistent with the discovery of the present invention that  
5 changes in sialylation are exquisitely pH sensitive: addition of even low concentrations of ammonium chloride to normal (corrected CF) cells caused defective sialylation and increased PNA binding, while a similar treatment of CFTR-mutant cells reversed the hyperacidification and under-sialylation defects.

As previously demonstrated (Bradbury, N. A. (1999) *Physiol Rev* 79: S175-S191; Barasch, J. *et al.* (1991) *Nature* 352: 70-3, 18-21; Imundo, L. *et al.* (1995) *Proc Natl Acad Sci USA* 92: 3019-3023; Saiman, L. and Prince, A. (1993) *J Clin Invest* 92: 1875-1880; Krivan, H. C. *et al.* (1988) *Proc Natl Acad Sci USA* 85: 6157-6161; and Dosanjh, A. *et al.* (1994) *Am J Physiol* 266: C360-C366), the decreased sialylation in CF leads to increased bacterial adhesion. Additionally, it is contemplated that hyperacidified  
15 endosomes and potentially other cellubrevin containing compartments in CF interfere with endosomal functions. (J. Biwersi, A. S. and Verkman (1994) *Am J Physiol* 266, C149-C156). Of particular interest are the repercussions on the secretory pathway and exocytosis, as membrane flow to the points of bacterial entry is important for phagocytosis (Hackam, D. J. *et al.* (1998) *Proc Natl Acad Sci U S A* 95, 11691-11696).  
20 It is contemplated that delivery of membrane to the nascent phagosome is obstructed, which would interfere with the efficient uptake of microorganisms by epithelial cells and their subsequent elimination (Pier, G. B. *et al.* (1996) *Science* 271, 64-67).

Furthermore, it is also contemplated that hyperacidified endosomes and potentially other cellubrevin containing compartments in CF result in increased inflammation.  
25 DNA from bacteria has stimulatory effects on mammalian immune cells, which depend on the presence of unmethylated CpG dinucleotides in the bacterial DNA (Hemml *et al.* (2000) *Nature* 408: 740-745). Most recently, it has been reported that acidification of endosomal CpG DNA is coupled to the rapid generation of intracellular reactive oxygen species, followed by NF-kB activation. (Hemml *et al.* (2000) *Nature* 408: 740-745)  
30 This supports the discovery of the present invention, and the role of hyperacidified endosomes in CF resulting in increased inflammation. CpG-mediated cell activation appears to require that the CpG must first be taken up by nonspecific endocytosis, as such activation is abolished by reagents such as bafilomycin A or chloroquine (Hemml *et al.* (2000) *Nature* 408: 740-745). Although these reagents are reported to inhibit



endosomal maturation (Hemml *et al.* (2000) Nature 408: 740-745), they may also function to reverse the hyperacidity of the endosomal compartment, thus preventing NF- $\kappa$ B activation and resulting inflammatory response, in accordance with the discoveries of the present invention.

5 The discoveries of the present invention provide a physiological link between the CFTR defect via organellar hyperacidification and the downstream effects of such hyperacidity, including respiratory pathogenesis and associated inflammation in CF. The discoveries of the present invention indicate that correction of the hyperacidity defect can be achieved by using an alkalinizing agent. Although it is not necessary to  
10 understand the mechanism in order to use the present invention, and it is not intended that the invention be so limited, it is contemplated that by decreasing the hyperacidity of the TGN and endosomes, sialylation is returned to normal levels, resulting in decreased bacterial adhesion and colonization of lung and tracheal epithelial cells. It is further contemplated that by decreasing the hyperacidity of the TGN and endosomes, efficiency  
15 of microorganism uptake and elimination by lung and tracheal epithelial tissues is increased, resulting in decreased infection. It is further contemplated that by decreasing the hyperacidity of the TGN and endosomes, associated inflammation is also decreased, as for example by decreasing or preventing activation of NF- $\kappa$ B..

Thus, the present invention provides methods of treating CF by normalizing the pH  
20 of the TGN and endosomes, such as by administering pH-normalizing chemicals and other drugs. The present invention also provides methods of screening reagents for their effectiveness in treating CF by examining their ability to normalize the pH of the TGN and endosomes in CF cells in vitro.

#### 25 A. Therapeutic Methods

The present invention provides a method of decreasing the hyperacidity of the TGN and endosomes of CF cells, and thereby reversing the effects of such hyperacidity. When used therapeutically, decreasing the hyperacidity of the TGN and endosomes of CF cells present in respiratory tissue results in ameliorations of infection and  
30 inflammation of respiratory tissue.

##### Cells

In one embodiment, a method of the present invention comprises administering an alkalinizing agent to at least one cell with a CF phenotype, under conditions sufficient to decrease the hyperacidity of the TGN and/or the endosomes of the cell. In one aspect of

the present invention, the under-sialylation of the cell is reversed. In another aspect, adherence of pathogenic bacteria to the cell is decreased. In yet another aspect of the invention, efficiency of uptake and elimination of pathogenic bacteria by the cell is increased. In yet another aspect of the present invention, inflammation is decreased. In one embodiment, cells are treated *in vitro*. In another embodiment, cells are treated *in vivo*. In another aspect, cells are respiratory epithelial cells. In yet another aspect, cells are pancreatic or epithelial cells.

In another embodiment, a method of the present invention comprises administering a physiologically tolerable therapeutic composition comprising an alkalinizing agent to a respiratory airway of an individual with CF, under conditions sufficient to decrease the hyperacidity of the TGN and endosomes of the respiratory epithelial cells. In one aspect of the present invention, the under-sialylation of the respiratory epithelial cells is reversed. In another aspect, adherence of pathogenic bacteria to the respiratory epithelial cells is decreased. In yet another aspect, efficiency of uptake and elimination of pathogenic bacteria by the respiratory epithelial cells is increased. In yet another aspect, inflammations of the respiratory epithelial cells is decreased.

The respiratory airways comprise a large and complex collection of organs, that encompasses all of the tissues having surfaces exposed to the passage of air during normal breathing through either the nose or mouth. Thus, the respiratory airways include air-exposed surfaces of the nasal passage, larynx, mouth, trachea, lung bronchi, lung bronchioles, lung alveolar ducts, lung alveolar sacs and lung alveoli, although the lung and associated organs are the primary target for infection and inflammation. Thus, the therapeutic composition comprising an alkalinizing agent according to the present invention can be administered to any or all of the affected tissues of the respiratory airways, although the typical and primarily infected and inflamed airway is the lung, and the associated ducts, sacs and alveoli.

#### Therapeutic Compositions

A therapeutic composition for use in the methods of the present invention comprises a therapeutically effective amount of at least one alkalinizing agent; an alkalinizing agent is thus an active ingredient. A therapeutic composition is preferably also physiologically tolerable. An alkalinizing agent is any chemical compound, regardless of size, biological activity, mode of action, etc., which, upon contacting a cell, effects a reduction in the pH of at least the TGN or the endosomes of the cell. As described in the Examples, it was observed that the TGN and the endosomes of CF cells are hyperacidic

relative to the TGN and endosomes of corrected CF cells; the difference in pH varies and is in the range of 0.1 to 1.0 pH units. It was also observed that CF cells are undersialylated, as determined by PNA binding, and as has been previously reported. Thus, an alkalinizing agent can be any reagent which can reduce the pH of the TGN or of the endosomes or preferably both; preferably, the pH should be reduced to about the optimal range of sialyltransferases. An alkalinizing agent is preferably one of a few types. One type comprises basic compounds which act directly by reducing the pH chemically. Another type comprises pharmacological agents which perturb cellular function to inhibit or decrease acid production in the TGN or the endosomes or both, or to promote base formation in the TGN or endosomes or both, such that pH is increased. Yet another type is an exogenous factor introduced by a heterologous gene, which acts to inhibit or decrease acid production in the TGN or endosomes or both, or to promote base formation in the TGN or endosomes or both, such that pH is increased.

Preferably, a basic compound is a weak base; a weak base refers to the state of association and dissociation of protons with the base, such that at physiological pH the base is capable of existing in both states in an equilibrium; the dissociated form is capable of moving slowly from an external environment into a subcellular compartment, passing the subcellular membrane in an uncharged form, and in an acidic subcellular compartment is capable of being protonated where it is essentially trapped. Preferably, a weak base is a compound which will accumulate in acidic cellular compartments and has the capacity to normalize the pH of the compartment by neutralizing an acidic pH to a more physiological pH. Examples of organic bases include but are not limited to primary, secondary, and tertiary amines (such as ammonia), chloroquine, and bicarbonate.

Basic compounds are available from a variety of commercial sources such as Sigma Chemical Corp, (St. Louis, Mo.). A base is used at concentrations effective to achieve a decrease in hyperacidification of TGN or endosomes or both to approximately physiological pH; such concentrations vary, but typically are present in a therapeutic composition of from about 0.01 millimolar (mM) to about 100 mM. Typically, a base is present in the composition at a physiological or weakly basic pH, such as from about 6.5 to about 8.

Alkalinizing agents also include pharmacological agents which perturb or modulate cellular function to inhibit or decrease acid production in the TGN or endosomes or both, or to promote base formation in the TGN or endosomes or both, such that PH is

increased. For example, in one aspect, such agents target  $\text{Na}^+$  channels, proton ATPases, or  $\text{Na}^+/\text{K}^+$  ATPases. In one embodiment, pharmacological agents which affect  $\text{Na}^+$  channels inhibit  $\text{Na}^+$  conductance, preferably resulting in an open channel such that a  $\text{Na}^+$  efflux from the TGN occurs, resulting in an increase in the pH. One  
5 example of such an agent is amiloride. In another embodiment, pharmacological agents inhibit proton ATPases; such agents include but are not limited to bafilomycin A, omeprazole and anti-acids, including but not limited to lansoprazole and omeprazole. Such agents may be chemically modified, in order to modify toxicity and in order to modify activity relative to inhibiting proton ATPases. In another embodiment,  
10 pharmacological agents modulate Na/K ATPases. Such agents enhance Na accumulation in the lumen of TGN, by activating Na/K ATPase, and thereby reversing acidification of the TGN.

Pharmacological agents are used at concentrations effective to achieve a decrease in hyperacidification of TGN or endosomes or both to approximately physiological pH;  
15 such concentrations are easily determined by routine experimentation, as for example by the screening assay described below. Typically, such concentrations range from 1 nM to 10 mM.

Therapeutic compositions of the present invention typically contain a physiologically tolerable carrier together with at least one species of alkalinizing agent as described herein, dissolved or dispersed therein as an active ingredient. In a preferred  
20 embodiment, the therapeutic composition is not immunogenic when administered to a human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and  
25 reagents, are used interchangeably and represent that the materials are capable of administration to or upon a subject or patient, preferably a mammal, most preferably a human, and that do not substantially produce, for example, adverse or allergic reactions when administered to a subject or patient, or that the materials can be administered without the production of undesirable physiological effects such as nausea,  
30 dizziness, gastric upset, toxicity and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile formulations either as liquid solutions or suspensions, aqueous or nonaqueous; however, solid forms suitable for solution or suspensions in liquid prior to

use can also be prepared. The preparation can also be emulsified. Thus, an alkalinizing agent-containing composition can take the form of solutions, suspensions, aerosols, or other compositional forms.

The active ingredient or ingredients can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein.

Physiologically tolerable carriers are well known in the art. Examples of liquid carriers include but are not limited to sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol, and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Examples of such additional liquid phases include but are not limited to glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

#### Therapeutically Effective Amounts

##### *In vitro*

A therapeutically effective amount of an alkalinizing agent is the amount or concentration of alkalinizing agent which, when contacted with a CF cell, results in a decrease of the hyperacidification of the TGN or endosomes, or both, to about the level observed in normal cells or corrected CF cells; this level is also referred to as approximately normal physiological levels, and represents a pH range of approximately about 6.5 to 6.8. A therapeutically amount can be determined as described below in the screening methods of the present invention.

*In vivo*

The dosage ranges for the administration of an alkalinizing agent of the invention are those large enough to produce the desired effect of decreasing the hyperacidity of the TGN or endosomes or both to approximately normal physiological levels. An effective amount is an amount which reduces the hyperacidity of the TGN or endosomes or both, in other words, a hyperacidity reducing amount. This reduction in hyperacidity is determined indirectly *in vivo*, by a number of different diagnostic procedures, as described further below; these diagnostic procedures include an improvement in pulmonary function as determined by any of several tests, and a reduction in infections and in inflammations when compared to untreated individuals.

The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication.

In particular, a therapeutically effective amount is a hyperacidity-reducing amount of at least one alkalinizing agent. Reduction in hyperacidity has a number of effects, including increased sialylation; when administered to lung tissue, reduction in hyperacidity results in decreased lung tissue infection by pathogenic bacteria, decreased lung tissue inflammation, and increased lung function. A hyperacidity-reducing amount can be determined in a variety of ways. For example, application of an alkalinizing agent to a patient with CF reduces the hyperacidity if any of a variety of measurable changes is observed in the patient or in the patient's lungs.

For assessing the effectiveness of the therapeutic compositions described above an individual with CF, indirect assay methods are available. Such indirect assays include measuring the degree of sialylation of glycoproteins in the sputum, analyzing bacterial content of lung tissue, evaluating physiological measurements of lung capacity, and determining qualitative aspects of well-being as noted by the patient and/or the physician.

A preferred indirect assay method is based on the detection of sialylated glycoproteins in the sputum sample after treatment. CF patients are characterized as having reduced sialylation with N-acetylneuraminic acid (sialic acid) of glycoproteins contained within the mucous. Changes of sialylation can be measured by reacting a sputum sample, either in solution or as immobilized, with a sialic acid binding lectin, such as peanut lectin or wheat germ agglutinin, where the lectins are labeled with a

detectable label such as biotin or a radioactive label. Depending on the label used, the sample can be read directly or may require additional marking before being read. One such additional marking is subsequent immunoreaction, such as with biotin reacting with avidin labeled with detectable reagent such as horseradish peroxidase. The amount of labeled sample is compared between the untreated and post-treated samples to assess an alteration of sialylation and thereby the effectiveness at alkalinizing intracellular compartments. Other sialic acid-binding lectins can also be used in this assay as well as others known to those of ordinary skill in the art of glycoprotein biochemistry.

Thus, a preferred method for determining a hyperacidity-decreasing amount of a therapeutic composition comprises measuring the amount of sialic acid on the mucous glycoproteins before and after a treatment, wherein an effective amount would be the amount sufficient to induce about at least about a 10% increase, preferably about a 30% increase, and most preferably about a 2-3 fold increase, in the amount of sialylation in the mucous glycoproteins. Methods for determining sialic acids in glycoproteins are well known and not considered to be limiting.

Another indirect assay to determine the efficacy of therapeutic treatment is to measure the amount bacterial content by standard microbiological techniques in sputum samples before and after treatment.

Yet another indirect assay to determine the efficacy of therapeutic treatment is to measure the amount of inflammation in the tissue.

Another preferred indirect assay for measuring effectiveness of the therapeutic compositions described above are physiologic pulmonary function tests. Such tests include but are not limited to spirometry (which determines the amount of air breathed and the speed at which it is blown out), determination of lung volumes, which can be measured by helium dilution method (which can determine the total lung capacity and residual volume) or by total body plethysmography, measurement of body gasses (from a blood sample or by oximetry), stress tests (as for example by exercise), and chest X-rays (which detect the condition of the lungs). For example, measurements of forced expiratory volume and forced vital capacity are taken before and after treatment with at least one alkalinizing agents. The pulmonary lung function test parameters and methods are well known to one of ordinary skill in the art of physiologic lung measurements.

In addition to direct pulmonary measurements, dyspnea, or the shortness of breath, can also be visually assessed and scaled before and after treatment by either the clinician or the patient. The bottom of the scale represents no shortness of breath while the top

represents severe shortness of breath. These results can be correlated with visual results of chest X-rays in which degrees of congestion and of open airways can be detected.

Lastly, another preferred method for assessing treatment efficacy is by a quality of life determination based on aspects of well-being including feeling, energy, physical activity, appetite, sleep patterns, and on aspects of disease symptoms including ease of sputum expectoration, cough frequency, cough severity and congestion. The qualities are ranked on a five-point Likert scale in which 1 represents the worst symptoms and 5 represents no symptoms.

The assays described above can be used individually or combined in various modalities to provide for an accurate assessment of treatment efficacy in decreasing hyperacidity in intracellular compartments of CF cells.

#### Route of Administration

A therapeutic composition containing at least one alkalinizing agent of this invention is conventionally administered by contacting the composition with the cells in the respiratory airways.

The administration of therapeutic compositions to the respiratory airways is a well-developed art in the field, and such methods are applicable here. Typically, an aerosolized or nebulized (vaporous) liquid composition containing a therapeutically-effective amount of an acidifying agent is delivered to the respiratory airways by breathing in the vaporous composition, or by forced (pressurized) periodic inflation breathing of the lungs with the vapor.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the manner of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals. Alternatively, continuous delivery of an aerosolized or nebulized composition during continuous breathing sufficient to bathe the respiratory airways is contemplated.



Thus, the administration of the vaporous composition can be in the form of a single unit dose, multiple inhalations, or during continuous breathing. Alternatively, a lavage of the lungs may be utilized whereby the lavage solution contains the alkalinizing agent.

The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; in other words, carrier, or vehicle.

Means for delivering a therapeutic composition comprises a device which produces an aerosol of a liquid composition, which devices are generally well known in the art. These devices can be nebulizers, small particle aerosol generators, inhalers with a propellant, and the like devices.

An exemplary nebulizer includes an Acorn II jet nebulizer, a Marquest or DeVilbiss 646 nebulizer, a compressed air generator such as a Pulmaide, a DeVilbiss, or an IPPB device, which typically nebulize 3-10 milliliters (ml) of solution over about 5-20 minutes, and the like commercially available nebulizers. Aerosol droplets produced by nebulizers are typically of a size that deposits the aerosolized droplets in the larger bronchioles of the lung.

Alternatively, a small particle aerosol generator, such as the commercially available SPAG-2 or Viratek, generates smaller droplets which are deposited more distally in the airways, such as in the ducts and sacs.

The duration and frequency of the administration of a therapeutic composition can vary widely depending upon the severity of the symptoms and infectious state. Typical dosages can be from one unit dose up to a continuous contacting dose over a period of from one to several days. Thus, the contacting can follow a variety of regimens. Exemplary regimens include one or more brief, unit dose, administrations over time to continuously inhaled aerosols for prolonged periods of from 5 minutes up to several hours or even days. More frequent administrations may be used under conditions of rapid cell turnover, such as during infection.

The present invention contemplates reducing hyperacidification by intranasal administration and/or respiratory inhalation of specific compounds. In some embodiments, intranasal administration and respiratory inhalation are the preferred modes of administration due to the ease of administration and faster onset of therapeutic activity. It is contemplated that intranasal administration and respiratory inhalation are

advantageous as they may allow a smaller effective dosage to be administered than would be possible with the oral route of administration. A preferred mode of administration comprises administration to the lung. Intrapulmonary delivery of pharmacologic agents to patients can be accomplished via aerosolization. Alternatively, 5 the agent may be administered to the lung through a bronchoscope. Of course, the therapeutic agents may be investigated for their efficacy via other routes of administration, including parenteral administration.

### B. Screening Methods

10 The present invention also provides screening methods of assaying compounds for determining effectiveness of compounds in decreasing the hyperacidity of the TGN or the endosomes or both of CF cells, and thereby reversing under-sialylation of CF cells, as well as increased infection and inflammation associated with CF. In one embodiment, the method comprises providing a CF cell transformed with a marker which is targeted 15 to at least one intracellular compartment and which is a pH indicator, contacting the cell with a test compound, and measuring the effect of the test compound on the pH of the targeted intracellular compartment. Preferably, the method further comprises providing at least one second control cell, where the second cell is a non-CF or a CF-corrected cell, where the second cell is transformed with a marker which is the same as the marker 20 targeted to at least one intracellular compartment for the first cell, measuring in the second cell the pH of the intracellular compartment to which the marker is targeted, and comparing the intraorganellar pH of the second cell to that of the first cell, both before and after contacting the first cell with the test compound.

A CF cell is one which comprises a mutation in the *CFTR* gene, and which when 25 present in a subject, results in symptoms of CF. Preferably, the cell is a cultured cell, derived from an established cell line. Examples of CF cell lines include but are not limited to IB3-1, CFT1, CFT1-LC3 and CFT1-508.

A non-CF cell is a cell which does not possess a mutation in the *CFTR* gene which results in cystic fibrosis. A corrected CF cell is a cell which possess a mutation in a 30 *CFTR* gene which results in cystic fibrosis, but in which the defect has been corrected; typically, such correction is by transformation of the cell with a normal *CFTR* gene, but other corrections are also contemplated. Preferably, the cell is a cultured cell, derived from an established cell line. Examples of corrected cell lines include but are not limited to C-38 and S-9, which are stably transfected and fully characterized derivatives

of IB3-1 cells corrected for chloride conductance by introduction of a functional *CFTR* gene (Egan, M. *et al.* (1992) *Nature* 358: 581-584).

A marker which is targeted to at least one intracellular compartment and which is a pH marker includes fusion constructs, in which one part of the construct is a first protein normally localized within a particular organelle, or localized to a membrane of an organelle, oriented in such a way such that the pH indicator is exposed to the lumen of the intracellular compartment. Preferably, the intracellular compartments to be examined are the TGN and the endosomes. Preferably, the first protein is cellubrevin, TGN38, or glycosylphosphatidylinositol (GPI), which are targeted to the endosomes, the TGN, and the plasmalemma, respectively. Another part of the construct is a second protein which possesses some characteristic which can be detected, and which changes in response to a change in pH, where the amount of the change is correlated with the amount of the change in pH and where the amount of the change in the characteristic can be measured. Preferably, the second protein is pHluorin green fluorescent protein (GFP). Preferably, the constructs are cellubrevin-pHluorin GFP, TGN38-pHluorin GFP, and GPI-pHluorin GFP.

The method may further comprise transforming the first cell or the second cell or both cells with at least one of the markers. Transformation techniques are well known in the art; one example is described in Example 1B.

A test compound is a potential alkalinizing agent whose effects on the intracellular compartment are to be measured. Test compounds are prepared in concentrations ranging from about 1 nM to 100 mM. Preferably, test compounds are dissolved in solution, but they may be prepared in other forms, such as emulsions, encapsulated (as in lipidic vesicles), or suspensions. The solution may be water alone, or comprise additional compounds such as buffering agents and cell protectants (for example, to regulate osmolarity, such as glycerol or sugars, or to protect against proteases, such as BSA).

The cell is contacted with the test compound by exposing the cell to the test compound, preferably in solution. Any number of convenient means may be employed; the cells may be mixed with the test compound, or the cells may be exposed by means of an aerosol or vapor. Preferably, the cell is mounted in a perfusion chamber and incubated in a buffer comprising the test compound.

The effect of the test compound on the pH of at least one intracellular compartment is then determined by measuring the characteristic of the marker; typically, such

measurements are made over a period of time, either continuously or at discrete intervals. Typically, the time is from a few seconds to 24 hours or more. When the markers cellubrevin-pHluorin GFP, TGN38-pHluorin GFP, and GPI-pHluorin GFP are utilized, measurements of fluorescence are made by fluorescence microscopy. The ratio of emission at 508 nm upon excitation at 410 nm vs. 470 nm is obtained by using filter sets, as for example as described in the Example 1D.

The pH of the at least one intracellular compound of a second, control cell is measured under conditions identical to those described above for measuring the effect of the test compound in the first cell, except that the second control cell is not exposed to the test compound.

It is contemplated that the initial pH of the TGN, endosomes, or both, will be approximately 5.9 to 6.3. Those compounds which result in a decrease of the initial pH by at least about 0.2 pH units are considered effective compounds.

It is further contemplated that the screening methods of the present invention can be adapted to high through-put screening methods.

## EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, and in the preceding description, the following abbreviations apply: N (normal); M (molar); mM (millimolar);  $\mu$ M (micromolar); mol (moles); mmol (millimoles);  $\mu$ mol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams);  $\mu$ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters);  $\mu$ l (microliters); cm (centimeters); mm (millimeters);  $\mu$ m (micrometers); nm (nanometers);  $^{\circ}$ C (degrees Centigrade); CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; GPI, glycosylphosphatidylinositol; ENaC, epithelial sodium channel; ER, endoplasmic reticulum; iNOS, inducible nitric oxide synthase; pHluorin-GFP, pH-sensitive green fluorescent protein; PBS, phosphate buffered saline; PNA, peanut agglutinin; TGN, trans-Golgi Network.

**EXAMPLE 1****Materials and Methods****A. Cells and tissue culture**

5 IB3-1 is a human bronchial epithelial cell line derived from a CF patient with a DF508/W1282X CFTR mutant genotype (Zeitlin, P. L. *et al.* (1991) *Am J Respir Cell Mol Biol* 4: 313-319). C-38 and S-9 are stably transfected derivatives of IB3-1 cells corrected for chloride conductance by introduction of a functional CFTR (Egan, M. *et al.* (1992) *Nature* 358: 581-584). The physiological levels of expression of CFTR and  
10 its functionality have been established for C-38 cells (Egan, M. *et al.* (1992) *Nature* 358: 581-584). IB3-1, C-38 and S-9 cells were maintained in LHC-8 Media (Biosource Int., Rockville, MD), 10% FBS and 50 U/ml Penicillin and 50 mg/ml Streptomycin (GibcoBRL, Life Technologies, Gaithersburg, MD). CFT1 (Olsen, J. C. *et al.* (1992) *Hum Gene Ther* 3: 253-266; and Lee, A. *et al.* (1999) *Am J Physiol* 277: L204-L217) is  
15 derived from the tracheal epithelium of a CF patient homozygous for the CFTR DF508 mutation. Stably transfected derivatives of CFT1 included CFT1-LCFSN, expressing the wild-type CFTR gene, CFT1-D508, transfected with DF508 mutant CFTR gene, and CFT1-LC3, the vector transfected control cells. CFT1 and derivative cells were grown in F12 media (GibcoBRL, Life Technologies, Gaithersburg, MD) supplemented with  
20 10 mg/ml insulin, 1 mM hydrocortisone, 1 nM triiodothyronine, 10 ng/ml cholera toxin (Sigma, St. Louis, MO, USA), 3.75 mg/ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor and 5 mg/ml transferrin (Collaborative Research Inc., Bedford, MA) (Olsen, J. C. *et al.* (1992) *Hum Gene Ther* 3: 253-266). All cells were grown in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

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**B. Transfections**

TGN38-pHluorin GFP, cellubrevin-pHlurin GFP, and glycosylphosphatidylinositol (GPI)-pHluorin GFP DNA constructs can be prepared as described by Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195. The expression construct for a 2,6-sialyltransferase  
30 (EC 2.4.99.1) Sttyr isoform carrying a c-myc tag at the C-terminus can be prepared as described by Ma, J. *et al.* (1997) *J Biol Chem* 272: 672-679. IB3-1 cells and its derivatives were seeded at 105 cells/ml on 25 mm cover slips in 6 well plates. At 40-60 % confluency cells were transfected with 1 mg/ml DNA using lipofectin (GibcoBRL, Life Technologies, Gaithersburg, MD) for 6 h at 37°C, 5% CO<sub>2</sub>. CFT1 cells and its

derivatives were seeded at 105 cells/ml on 25 mm cover slips in 6 well plates and grown in the medium without cholera toxin. At 90% confluency, cells were transfected with GenePorter (Gene Therapy Systems, San Diego, CA) with 2.5 mg/ml DNA for 4 h at 37°C, 5% CO<sub>2</sub>. For observation and further manipulation, transfected cells were  
5 mounted in a perfusion chamber after 48 h of expression (Harvard Instruments, Holliston, MA) set at 37°C.

### C. Colocalization Studies

Cells grown on glass slides to 60-70 % confluency were co-transfected with 1 mg of  
10 TGN38-pHluorin GFP and myc tagged a 2,6-sialyltransferase DNA using 10 ml Lipofectin. After 36 hours of expression, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% saponin for 5 min. Mouse monoclonal antibody (9E10) against c-myc (Santa Cruz Biotechnology, Santa Cruz, CA) was followed by goat anti-mouse secondary antibody conjugated to Alexa 568 (Molecular Probes, Eugene, OR).  
15 Glass slides were mounted using PermaFluor (Shandon, Pittsburgh, PA) and analyzed by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/emitter cube set 8300 (Chroma Technology Corp., Brattleboro, VT).

### D. Fluorescence microscopy and pH measurements

20 Fluorescence microscopy was carried out using an Olympus IX-70 microscope and Olympix KAF1400 CCD camera (LSR, Olympus, Melville, NY, USA). The ratio of emission at 508 nm upon excitation at 410 nm vs. 470 nm was obtained using previously described (Miesenbock, G. *et al.* (1998) Nature 394: 192-195) filter sets (Chroma Technology Corp., Brattleboro, VT) mounted in a Sutter filter wheel (Sutter  
25 Instruments, Novato, CA, USA) and controlled by Merlin program (version 1.89; LSR, Olympus, Melville, NY, USA). For the pH standard curve, two types of calibrations were carried out: (i) Cells transfected with GPI-pHluorin GFP were mounted in a perfusion chamber and incubated in buffer A: 25 mM HEPES (pH changing from 7.4 to 5.5), 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 30 mM Glucose at  
30 37°C. Fluorescence images were taken upon excitation at 410 and 470 nm (6 consecutive exposures). Three regions of interest were selected and the standard curve was plotted as averaged 410/470 ratio values for a given buffer pH. (ii) At the end of experiments, the pH-gradient was collapsed by incubating cells in 10 mM monensin, a Na<sup>+</sup>/H<sup>+</sup> exchange ionophore, and 10 mM nigericin, a K<sup>+</sup>/H<sup>+</sup> exchange ionophore, for 30

min at 37°C in buffer A at pH 7.4 or pH 5.5 and ratios of TGN-38-pHluorin GFP were recorded for internal standards. Sample pH was determined the same way as for the external standard curve.

#### 5 E. Inhibition studies and ion substitutions

For H<sup>+</sup>-ATPase inhibition, TGN38-pHluorin GFP transfected cells were incubated with 100 nM bafilomycin A1 (Sigma, St. Louis, MO) in buffer A at pH 7.4 for 2.5 h at 37°C. For Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition, TGN38-pHluorin GFP transfected cells were treated with 1 mM acetylstrophanthidin (Sigma, St. Louis, MO) in buffer A at pH 7.4  
10 for 2 h at 37°C. For ion substitution experiments, TGN38-pHluorin GFP transfected cells were incubated for 30 min in buffer A at pH 7.4 at 37°C. Sodium was replaced by washing cells three times in buffer A containing 119 mM LiCl instead of NaCl and incubating cells in this buffer at pH 7.4, 37°C for 30 min. Sodium was re-introduced by replacing LiCl solution with buffer A (pH 7.4).

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#### F. Lectin binding studies

Lectin binding was carried out according to the published procedures (Dosanjh, A. *et al.* (1994) *Am J Physiol* 266: C360-C366; and Poschet, J. F. *et al.* (2000) *Methods in Enzymol.*). Cells were grown in standard media on 18 mm sterile coverslips placed in  
20 12-well plates (Corning, Corning, NY) to 80-90% confluency at 37°C in 5% CO<sub>2</sub>. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) containing 5% sucrose, followed by incubation for 2 h at 37°C with either PBS or 0.4 U/ml neuraminidase type X (Sigma, St Louis, MO) in 0.1 M acetate buffer pH 5.0. TRITC-conjugated Peanut Agglutinin (PNA) 0.1 mg/ml (Sigma, St Louis, MO) in PBS was  
25 added for 30 min at 37°C, 5% CO<sub>2</sub>. Coverslips were mounted with PermaFluor and examined by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/emitter cube set 8300 (Chroma Technology Corp., Brattleboro, VT). Relative fluorescence units were obtained by selecting 10 distinct regions of interest (individual cells) and subtracting the background fluorescence. Relative fluorescence was  
30 converted into %-binding with IB3-1 and CFT1 cells representing 100% binding.

### G. pH normalization treatment

Cells were grown for 48 h in complete LHC-8 media in the presence of 0.1-1.0 mM NH<sub>4</sub>Cl, chloroquine and lansoprazole (all from Sigma, St Louis, MO) at 37°C in 5% CO<sub>2</sub>. Staining, visualization and pH measurements were as describe above.

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### H. Statistics

All statistical analyses were carried out using Fisher's Protected LSD post hoc test (ANOVA) (SuperANOVA v1.11, Abacus Concepts, Inc., Berkeley, CA).

10

## EXAMPLE 2

### Hyperacidification of TGN and Endosomes in CF cells

For determination of organellar pH in live bronchial epithelial cells, the recently developed pH-sensitive GFP (pHluorin GFP) system for ratiometric determination of the luminal pH in intracellular organelles (Miesenbock, G. *et al.* (1998) Nature 394: 192-195) was employed. Three fusion constructs were used: TGN38-pHluorin GFP, and cellubrevin-pHluorin GFP, which target the TGN and cellubrevin-containing endosomes, respectively, and which both have lumenally exposed pH sensitive GFP, and GPI-pHluorin GFP, which targets the plasma membrane, and which exposes pHluorin GFP to the extracellular fluid (Miesenbock, G. *et al.* (1998) Nature 394: 192-195).

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The TGN-, endosome-, and plasma membrane-targeted pHluorin GFP probes were transfected into the well characterized human bronchial epithelial cells IB3-1, C-38 and S-9 (Zeitlin, P. L. *et al.* (1991) Am J Respir Cell Mol Biol 4: 313-319; and Egan, M. *et al.* (1992) Nature 358: 581-584), which have been used as standard cell lines to model the effects of CFTR mutations (Zeitlin, P. L. *et al.* (1991) Am J Respir Cell Mol Biol 4: 313-319; Egan, M. *et al.* (1992) Nature 358: 581-584; Schwiebert, E. M. *et al.* (1995) Cell 81: 1063-1073; DiMango, E. *et al.* (1998) J Clin Invest 101: 2598-2605; and Rubenstein, R. C. and Zeitlin, P. L. (2000) Am J Physiol Cell Physiol 278: C259-C267). IB3-1 is derived from a CF patient with a DF508/W1282X CFTR mutant genotype (Zeitlin, P. L. *et al.* (1991) Am J Respir Cell Mol Biol 4: 313-319). C-38 and S-9 are stably transfected and fully characterized derivatives of IB3-1 cells corrected for chloride conductance by introduction of a functional CFTR gene (Egan, M. *et al.* (1992) Nature 358: 581-584).

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Using immunofluorescence microscopy of fixed cells, TGN38-pHluorin GFP showed colocalization with myc tagged a-2,6-sialyltransferase Sttyr (Saiman, L. and Prince, A. (1993) *J Clin Invest* 92: 1875-1880) in cotransfected cells (Krivan, H. C., *et al.* (1988) *Proc Natl Acad Sci U S A* 85: 6157-6161). The endosomal localization of cellubrevin-pHluorin GFP was confirmed by colocalization with the exogenously added endocytic tracer dextran-Texas Red using fluorescence microscopy of live cells (Krivan, H. C., *et al.* (1988) *Proc Natl Acad Sci U S A* 85: 6157-6161).

The fluorescence appearance of GPI-pHluorin GFP at pH 7.4 and pH 5.5 was determined. A standard curve was generated within the working range of pHluorin GFP (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195) (pH 7.4 - 5.5) using IB3-1, C-38 and S-9 cells. All cells showed identical fluorescence dependence of the GPI-pHluorin GFP on pH of the external buffer. In addition, TGN38-pHluorin GFP transfected cells were treated at the end of each experiment with monensin and nigericin to generate internal standards for pH calibration.

The pH of TGN was probed with TGN38-pHluorin GFP (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195). The fluorescence of TGN38-pHluorin GFP transfected IB3-1 and C-38 cells upon excitation at 410 nm vs. 470 nm was observed, and the results presented in Table 1. The apparent pH of TGN38-pHluorin GFP compartment in C-38 cells was  $6.6 \pm 0.1$  (mean  $\pm$  SE, n=18) and in S-9 cells  $6.7 \pm 0.1$  (mean  $\pm$  SE, n=24), whereas the corresponding apparent pH in IB3-1 CFTR mutant cells was  $6.0 \pm 0.1$  (mean  $\pm$  SE, n=17) (Table 1). Thus, the internal pH-values of the TGN in IB3-1 cells were 0.7 and 0.6 pH-units lower than in C-38 and S-9 cells, respectively (P=0.0001), indicating that the TGN is hyperacidified in CF bronchial respiratory epithelial cells.

Next, IB3-1, C-38 and S-9 cells, transfected with pHluorin GFP fused to the lumenally exposed carboxy-terminal end of the endosomal R-SNARE cellubrevin (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195) were used to determine the pH of cellubrevin-containing endosomal compartments. The difference in fluorescence between cellubrevin-pHluorin GFP transfected IB3-1 and C-38 cells upon illumination at 410 nm vs. 470 nm was observed, and the results presented in Table 1. The pH of cellubrevin-containing endosomes was  $6.7 \pm 0.1$  (mean  $\pm$  SE, n=15) for the CFTR corrected C-38 and  $6.7 \pm 0.1$  (mean  $\pm$  SE, n=32) for S-9 cells, compared to the pH of IB3-1 CFTR mutant cells, which was  $6.2 \pm 0.1$  (mean  $\pm$  SE, n=19). To demonstrate that the differences in pH were due to organelle acidification, the pH-gradient was collapsed with monensin and nigericin. Upon pH-gradient collapse, the endosomal pH changed to

7.2, approaching the pH of the incubation buffer. Thus, cellubrevin labeled compartments in CF mutant cells show hyperacidification of a 0.5 pH unit ( $P=0.0001$ )

The observations that TGN and cellubrevin endosomes are hyperacidified in CF cells were confirmed using another well-characterized CF cell line, CFT1 (J. C. Olsen, *et al.* (1992) *Hum Gene Ther* 3: 253-266), derived from the tracheal epithelium of a CF patient homozygous for the DF508 mutation in CFTR. CFT1, and its stably transfected derivatives, CFT1-LCFSN, expressing the wild-type CF gene, CFT1-D508, expressing the DF508 mutant CFTR gene, and CFT1-LC3, serving as a control, were transiently transfected with TGN38- and cellubrevin-pHluorin GFP constructs. The CFTR-  
10 corrected variant CFT1-LCFSN had a pH of  $6.7 \pm 0.1$  in TGN compared to the CFTR mutant parental cell line CFT1 (DF508/DF508) with a pH of  $6.3 \pm 0.1$ , CFT1-D508 (DF508 CFTR transfected control) with a pH of  $6.2 \pm 0.1$  (Table 1) and CFT1-LC3 (vector transfected control) with a pH of  $6.3 \pm 0.1$ . The cellubrevin-pHluorin GFP compartment in the CFTR-corrected variant CFT1-LCFSN had a pH of  $6.6 \pm 0.03$   
15 compared to CFT1 (pH  $6.1 \pm 0.1$ ), CFT1-D508 (pH  $6.2 \pm 0.1$ ) and CFT1-LC3 (pH  $6.0 \pm 0.1$ ) (Table 1). Thus, the TGN38 and cellubrevin labeled compartments showed hyperacidification of 0.4 and 0.5 pH units in CFTR mutant cells.

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**Table 1**  
**Hyperacidification of TGN38 and cellubrevin -endosomal compartments**  
**in CF cells**

| 5  | Cell Line <sup>a</sup> | Endosomes <sup>b</sup>                | TGN38 <sup>c</sup>                      |
|----|------------------------|---------------------------------------|---|
|    | IB3-1 (mutant)         | pH 6.2 $\pm$ 0.1 (n=19) <sup>d</sup>  | pH 6.0 $\pm$ 0.1 (n=17) <sup>d</sup>    |
|    | C-38 (corrected)       | pH 6.7 $\pm$ 0.1 (n=15) <sup>d</sup>  | pH 6.6 $\pm$ 0.1 (n=18) <sup>d</sup>    |
|    | S-9 (corrected)        | pH 6.7 $\pm$ 0.1 (n=32) <sup>d</sup>  | pH 6.7 $\pm$ 0.1 (n=24) <sup>d</sup>    |
| 10 | CFT1 (mutant)          | pH 6.1 $\pm$ 0.1 (n=15) <sup>d</sup>  | pH 6.2 $\pm$ 0.1 (n=10) <sup>d, e</sup> |
|    | CFT1-LCFSN (corrected) | pH 6.6 $\pm$ 0.03 (n=14) <sup>d</sup> | pH 6.7 $\pm$ 0.1 (n=9) <sup>d, e</sup>  |
|    | CFT1-LC3 (mutant)      | pH 6.0 $\pm$ 0.1 (n=15) <sup>d</sup>  | pH 6.3 $\pm$ 0.1 (n=10) <sup>d, e</sup> |
|    | CFT1-DF508 (mutant)    | pH 6.2 $\pm$ 0.1 (n=19) <sup>d</sup>  | pH 6.2 $\pm$ 0.1 (n=19) <sup>f</sup>    |

15 <sup>a</sup> IB3-1 is a human bronchial cell line derived from a CF patient with a DF508/W1282X CFTR mutant genotype (Zeitlin, P. L. *et al.* (1991) *Am J Respir Cell Mol Biol* 4: 313-319). C-38 cells express a functional CFTR with a fortuitous N-terminal. S-9 cells express a functional full size CFTR (Schwiebert, E. M. *et al.* (1995) *Cell* 81: 1063-1073). CFT1 (Olsen, J. C. *et al.* (1992) *Hum Gene Ther* 3: 253-266) is a  
20 tracheal cell line derived from a CF patient carrying mutant DF508/DF508 CFTR gene. CFT1-LCFSN (wild-type CFTR), CFT1-D508 (D508 CFTR) and CFT1-LC3 (vector control) are stably transfected derivatives of CFT1.

<sup>b, c</sup> Mean value $\pm$ SE.

<sup>d</sup> P=0.0001 for IB3-1 vs. C38; IB3-1 vs. S-9; CFT1-LCFSN vs. CFT1-LC3; CFT1-  
25 LCFSN vs. CFT1-D508; CFT1-LCFSN vs. CFT1; CFT1-LCFSN vs. CFT1-D508

<sup>e</sup> P=0.0003 for CFT1-LCFSN vs. CFT1-LC3 and CFT1-LCFSN vs. CFT1

### EXAMPLE 3

#### 30 **Hyperacidification of TGN and endosomes in CF cells is sodium dependent**

The absence of a chloride channel activity of CFTR in CF cells cannot readily explain the organellar hyperacidification observed in our experiments. Instead, an alternative model is proposed, in which Na<sup>+</sup> efflux from the organelle could play a role, with a net effect similar to the influx of Cl<sup>-</sup>, in luminal charge neutralization.

In CF bronchial epithelial cells, the epithelial sodium channel ENaC is under negative regulation by CFTR. In the absence of CFTR, ENaC is relieved from CFTR inhibition, leading to an increase in Na<sup>+</sup> transport (Bradbury, N. A. (1999) *Physiol Rev* 79: S175-S191; Stutts, M. J (1995) *Science* 269: 847-850; Schreiber, R. *et al.* (1999) *Proc Natl Acad Sci USA* 96: 5310-5315; Ismailov, II *et al.* (1996) *J Biol Chem* 271: 4725-4732; Stutts, M. J. *et al.* (1997) *J Biol Chem* 272: 14037-14040; and Kunzelmann, K. and Schreiber, R. (1999) *J. Membrane Biol* 168: 1-8).

To test the proposed model, in which altered sodium transport could play a role in TGN hyperacidification via effects on H<sup>+</sup>-ATPase activity in CF, a possible role of H<sup>+</sup>-ATPase in acidification of TGN and endosomes in CF cells was tested. Treatment with bafilomycin A1 abrogated hyperacidification of both TGN and cellubrevin-labeled endosomes in CFTR mutant cells.

Next the role of sodium was tested. Incubation in Na<sup>+</sup>-free buffers normalized the pH of both TGN and cellubrevin-labeled compartments in IB3-1 cells. Thus, after the substitution of NaCl with LiCl, the mean apparent pH of TGN in CFTR<sup>DF508/W1282X</sup> IB3-1 cells was increased by pH 0.6 $\pm$ 0.2 (P=0.002), reaching the values of normal cells, while the mean pH of cellubrevin labeled endosomes was increased to pH 6.7 $\pm$ 0.1, also reaching the values of normal cells. Reintroduction of NaCl at the end of the experiment restored hyperacidification of both TGN and the endosomes in IB3-1 (P=0.0038). Differences observed in acidification patterns between cellubrevin labeled endosomes and TGN in the ion substitution experiments most likely reflect differences in additional ion pumps and transporters present in these intracellular compartments. There was no significant change in pH of TGN upon sodium removal and replacement in both CFTR corrected cells (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195; and H. C. Krivan, H. C. (1988) *Proc Natl Acad Sci USA* 85: 6157-6161). These observations are in keeping with a participation of Na<sup>+</sup> conductances in hyperacidification of TGN in CF.

Additionally, the source of luminal Na<sup>+</sup> was examined. Treatment of cells with the Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor acetylstrophanthidin reduced the pH of CFTR-corrected cells to the levels in CF cells (P=0.0111), and, as expected, had no effect on TGN acidification in CFTR mutant cells (P=0.5170). These observations are consistent with a model in which Na<sup>+</sup>/K<sup>+</sup>-ATPase is a source of luminal Na<sup>+</sup> that causes differential TGN acidification in normal and CF cells.

**EXAMPLE 4****Normalization with weak bases corrects under-sialylation in CF cells**

The increased acidification of TGN and endosomes in CF cells may have significant consequences on glycosylation patterns. Similar to the alkalization hypothesis (Barasch, J. *et al.* (1991) *Nature* 352: 70-73), a hyperacidified TGN can lead to altered protein and lipid glycosylation, manifested as decreased sialylation, since sialyltransferases responsible for protein modification have bell-shaped pH-optima (Busam, K. and Decker, K. (1986) *Eur J Biochem* 160: 23-30).

To address this possibility, it was first determined whether the TGN38-pHluorin GFP pH probe was localized in a compartment relevant for sialyltransferases, by examining the distribution of TGN38-pHluorin GFP and myc-tagged  $\alpha$ -2,6 sialyltransferase Sttyr (Ma, J., *et al.* (1997) *J Biol Chem* 272: 672-679) in co-transfected cells. The results of these experiments demonstrated identical overall organellar distribution and a nearly complete overlap of these two markers. Next the sialylation of surface glycoconjugates was examined. Fluorescently labeled peanut agglutinin (PNA), which recognizes unsialylated galactosyl (b-1,3) N-acetylgalactosamine, bound better to IB3-1 (100 $\pm$ 3%, mean $\pm$ SE, n=30), CFT1 (100 $\pm$ 4%, mean $\pm$ SE, n=30), CFT1-D508 (99 $\pm$ 3%, mean $\pm$ SE, n=30), and CFT1-LC3 (97 $\pm$ 4%, mean $\pm$ SE, n=30) cells than to the CFTR corrected C-38 (35 $\pm$ 3%, mean $\pm$ SE, n=30), S-9 (55 $\pm$ 4%, mean $\pm$ SE, n=30) or CFT1-LCFSN (52 $\pm$ 3%, mean $\pm$ SE, n=30) cells (P=0.0001, CFTR mutants vs. CFTR corrected cells) in a process which was neuraminidase sensitive.

If hyperacidification of TGN is responsible or contributing to the under-sialylation of glycoconjugates in CF, then normalization of pH using weak bases may correct this defect. Different concentrations of ammonia concentrations were titrated, and it was discovered that when IB3-1 cells were treated with 0.1 mM ammonium chloride, the pH was corrected and matched the normal levels in CFTR-corrected C-38 cells (ammonia treated IB3-1 vs. C-38, P=0.7440). Additionally, treatment of IB3-1 cells with another weak base, chloroquine, also corrected the pH of TGN to normal levels (chloroquine treated IB3-1 vs. C-38, P=0.1066). In contrast, treatment of IB3-1 cells with the gastric proton pump inhibitor Lansoprazole did not have an effect on pH of TGN (P=0.7976).

Treatment for 48 h with ammonia at concentrations which repaired the hyperacidification defect (in other words 0.1 mM), also restored normal patterns of PNA binding in IB3-1 CFTR mutant cells, similar to those seen in CFTR-corrected cells. The levels of PNA binding in NH<sub>4</sub>Cl-treated IB3-1 cells were equal to those in

CFTR-corrected C-38 cells. Treatment of C-38 cells with 0.1 mM NH<sub>4</sub>Cl increased PNA binding, indicating a reduced sialylation in normal cells, consistent with a bell shape pH optimum for sialyltransferase activity (Busam, K. and Decker, K. (1986) Eur J Biochem 160: 23-30). The levels of PNA binding in ammonia treated C-38 cells were  
5 similar to the levels seen in untreated and aberrantly under-sialylated IB3-1 cells. These observations are in keeping with a requirement for an optimal pH in TGN in order for sialyltransferases to work properly. These experiments show that this optimum can be disturbed by conditions that are either too acidic (as in CF cells) or too alkaline (normal cells treated with ammonia). Furthermore, these findings indicate that pH correction of  
10 TGN in CFTR mutant cells can lead to a restoration of normal sialylation.

### EXAMPLE 5

#### **Hyperacidification of TGN and endosome depends upon CFTR mutations**

As described in the preceding Examples, the low pH in the TGN and endosomes of  
15 CF respiratory epithelial cells is ascribed to CFTR absence or dysfunction in corresponding intracellular compartments. Furthermore, correction of the defects in IB3-1 and CFT1 cells with normal CFTR restores normal pH.

The role of CFTR is confirmed by co-transfecting IB3-1 and CFT1 cells with TGN-38-pHluorin GFP or cellubrevin-pHluorin GFP and DsRed-CFTR constructs (DsRed  
20 fluoresces red at 583 nm with excitation maximum at 558 nm; 570/20 excitation filter and 620/60 emission-dichroic mirror cube is routinely used). DsRed CFTR and GFP-CFTR colocalize, thus showing that the two protein fusions traffic identically. Expression of DsRed-CFTR corrects hyperacidification in IB3-1 and CFT1 cells when they express DsRed-CFTR as opposed to a nontransfected (in other words, DsRed  
25 negative) neighboring cell. In addition, colocalization experiments of DsRed CFTR and TGN-38-GFP or cellubrevin-GFP carried out at different times post-infection using confocal microscopy indicate the extent that CFTR overlaps with these compartments. When necessary, the cells are treated with Brefeldin, (the half-life of CFTR on plasma membrane depending upon stimulation is between 6 and 22 h) to trap CFTR in the  
30 Golgi. Removal of Brefeldin A allows the observation of redistribution of CFTR and cellubrevin-GFP using fixed samples or in live cells using a VisTrack TILL technology setup (Tvarusko, W. (1999) Proc Natl Acad Sci USA 96: 7950-7955). The functionality of DsRed CFTR is indicated by monitoring cAMP-dependent (upon

stimulation with 10 mM forskolin and 200 mM IBMX) efflux of anions using halide-quenched fluorescent dye SPQ and cells perfused with NaI.

As an alternative, endogenous CFTR in IB3-1 and CFT1 cells is rescued by chemical chaperones, 25 mM 8-cyclopentyl-1,3-dipropylxanthine (CPX) (Al-Nakkash, L. and Hwang, T. C. (1999) *Pflugers Arch* 437: 553-561; Arispe, N. *et al.* (1998) *J Biol Chem* 273: 5727-5734; Srivastava, M. *et al.* (1999) *Mol Med* 5: 753-767; Haws, C. M. *et al.* (1996) *Am J Physiol* 270: C1544-C1555; and He, Z. (1998) *Am J Physiol* 275: C958-C966), 5 mM butyrate or phenylbutyrate (Zeitlin, P. L. (1999) *J Clin Invest* 103: 447-452), or by incubation at lower temperatures (25-29° C) (DiMango, E. *et al.* (1998) *J Clin Invest* 101: 2598-2605; Rubenstein, 2000 #174; Loffing, J. *et al.* (1999) *Am J Physiol* 277: L700-L708; and Moyer, B. D. *et al.* (1999) *Am J Physiol* 277: F271-F276). In yet another alternative, since IB3-1 cells have one W1282X CFTR allele, an aminoglycosides such as G418 or gentamicin is applied.

Restoration of normal pH, monitored by TGN-38-pHluorin GFP and cellubrevin-pHluorin GFP in CFTR co-transfected IB3-1 and CFT1 cells, or previously corrected stable cell line derivatives C-38, S9, and CFT1-LCFSN, confirms that the hyperacidification is linked to CFTR function and trafficking. The absence of CFTR is responsible for hyperacidification, and the pH of the corresponding organelles is normalized in CFTR-cotransfected cells or in cells with restored DF508 CFTR folding and trafficking.

## EXAMPLE 6

### Characterization of TGN and endosome hyperacidification

In the following experiments, fluorescence of pH-sensitive GFP (ratiometric pHluorin) is monitored as described above, using two microscopes set up for this type of analysis (one with Merlin/Esprit package and a Sutter wheel, and another with TILL software and controller along with a monochromator and piezzo Z-drive). Data is collected on at least 6 sequential acquisitions at 410 and 470 nm excitation wavelengths and emission at 508 nm (Miesenbock, G. *et al.* (1998) *Nature* 394: 192-195), and averaged to eliminate differential bleaching effects. At least 3 regions of interest are used to derive pH (this number can be increased without affecting speed due to a highly automated processing of the data).

**A. Effects of ions**

Effect of Sodium removal. Sodium is substituted with choline as follows: Cells (CFTR mutants and corrected) are transfected with cellubrevin-pHluorin GFP or TGN38-pHluorin GFP as described in the preceding Examples. After 48 h of expression, transfected cells are mounted in a perfusion chamber (Harvard Instruments) set at 37°C and the initial pH is recorded in at least five distinct cells in buffer A (25 mM HEPES (pH 7.4), 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 30 mM glucose). The ratio of emission at 508 nm upon excitation at 410 nm vs. 470 nm is obtained. Next, Na<sup>+</sup> is replaced by adding buffer A containing 119 mM choline chloride instead of NaCl and the pH is recorded every 15 minutes for up to 2 h. Finally, Na<sup>+</sup> is reintroduced by addition of buffer A, and the pH is recorded every 15 minutes for up to 2 h. The pH in mutant CFTR cells increases in the TGN and endosomes upon removal of sodium, and this increase is reversed upon the reintroduction of sodium, thus confirming dependence on Na<sup>+</sup>.

Effect of chloride removal. Cl<sup>-</sup> is removed by washing and incubating the cells in buffer B containing 25 mM HEPES (pH 7.4), 119 mM D-gluconic acid (Na<sup>+</sup> salt), 2.5 mM D-gluconic acid (K<sup>+</sup> salt), 2mM D-gluconic acid (Ca<sub>2</sub><sup>+</sup> salt), 2 mM D-gluconic acid (Mg<sub>2</sub><sup>+</sup> salt), and 30 mM glucose. The pH of TGN and endosomes is determined after 20, 60 and 120 min of incubation. In an alternative, CFTR mediated chloride conductance via cAMP is stimulated by forskolin (Al-Nakkash, L. and Hwang, T. C. (1999) Pflugers Arch 437: 553-561; Haws, C. M. *et al.* (1996) Am J Physiol 270: C1544-C1555; and Heda, G. D. and Marino, C. R. (2000) Biochem Biophys Res Commun 271: 659-664). In accordance with the discoveries of the present invention, removal of chloride ions does not affect acidification.

Effects of inhibitors of Na<sup>+</sup>/K<sup>+</sup> ATPase A membrane permeant inhibitor of Na<sup>+</sup>/K<sup>+</sup> ATPase, acetylthiocholine, which works on the Na<sup>+</sup>/K<sup>+</sup> ATPase present on endomembranes of compartments in which hyperacidification in CF cells, results in a decrease of hyperacidification. Ouabain has similar effects.

**B. The pH of endosomes and TGN in polarized CF cells**

Because IB3-1 and C-38 cells do not form tight junctions, these cells are not polarized. Therefore, the pH of TGN and endosomes in polarized cells, CFT1 and corrected CFT1 cells, is examined. CFT1 cells and their derivatives are seeded onto 0.4 mm transwell filters at a concentration of 106 cells/cm<sup>2</sup>. Polarized monolayers



exhibiting a resistance of about 100-200 cm<sup>2</sup> or higher are examined by confocal microscopy. In accordance with the discoveries of the present invention, the pH of the endosomes and TGN of polarized are hyperacidified.

### C. The pH in the TGN and cellubrevin endosomes of primary cells

5 The pH of the TGN and endosomes is also examined in primary cells by the methods described previously. Primary human bronchial cells from CF donors and from non-CF lungs are grown in Clonetics BEGM supplemented with Pen-Strep, gentamicin (should not affect DF508 homozygous cells), fluconazole and an attachment factor, human type IV placental collagen. For the first several days, the cells are grown  
10 in 12.5 mg/ml Primaxin, 70 mg/ml ceftazidime, and 2.5 mg/ml colistin. To optimize transfection reactions, cells are transfected with GenePORTER and Lipofectin, and then examined within 24 to 48 h of transfection. In the alternative, cells are transfected by microinjection, with an Eppendorf Transjector 5246 microinjector (Eppendorf, Westbury, NY) mounted on the microscope. Following microinjection, cells express the  
15 transfected DNA within 4 h. In another alternative. Alternatively, adenoviral or retroviral vectors are constructed with relevant DNA (for example Cellubrevin pHluorin-GFP). In accordance with the discoveries, the TGN and endosomes of primary human bronchial CF cells are hyperacidic, compared to those from non-CF lungs.

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## EXAMPLE 7

### Effects of hyperacidification on trafficking processes in respiratory epithelial cells

The following experiments examine whether organelle trafficking processes are altered in CF cells as a consequence of the hyperacidification of intracellular  
25 compartments.

### A. Localization of sialyltransferases in TGN and endosomes

Additional experiments confirm that sialyltransferases are overlapping with the compartments which are hyperacidified in CF cells. IB3-1, C-38, and S-9 cells are co-  
30 transfected with myc-ST (Ma, J. *et al.* (1997) *J Biol Chem* 272: 672-679) and TGN-38-pHluorin GFP at 60-70% confluency using 1 mg of each DNA and 10 ml Lipofectin. After 24-36 h of transfection, cells are fixed and permeabilized with 0.2% Saponin for 5 min and incubated in blocking solution for 30 min at room temperature. Mouse monoclonal antibody (9E10) against c-Myc (Santa Cruz) diluted 1:300 (Lu, B. *et al.*

(1998) Cell 95: 225-235) and secondary Goat anti-mouse antibody conjugated to Alexa 568 are used to visualize ST. Pre-treatment of cells with nocodazole or brefeldin prior to processing cause redistribution of TGN in the cell and colocalization is further demonstrated when the two markers (sialyltransferase and TGN38-GFP) remain  
5 overlapping.

In the case of cellubrevin-pHluorin GFP, in both fixed and live cells, localization relative to endocytic tracers such as fluid phase (Dextran-Texas Red) or receptor mediated endocytosis markers (for example transferrin) are examined. These experiments are carried out as follows on fixed cells: IB3-1, C-38, and S-9 cells are  
10 transfected with cellubrevin-pHluorin GFP, pulsed with Texas-Red-labeled Dextran (Mr 10,000) for 5 min at 37°C. The endocytic tracer is chased for varying periods of time to label early (5-10 min) or late (30-60) min endocytic compartments. For transferrin labeling, cells are preincubated in serum-free media to deplete endogenous transferrin, and plasma membrane transferrin receptor loaded on ice with iron-saturated, Texas red-  
15 conjugated human transferrin. IB3-1, C-38, and S-9 cells are transfected with cellubrevin-pHluorin GFP using 1 mg DNA for cells of 60-70% confluency and 10 ml Lipofectin (Gibco). After 24-36 h of transfection, the cells are washed with serum-free media (LHC-8 with 2% BSA and 20 mM Na HEPES). The cells are incubated in serum-free media at 37°C for 30 minutes, and then moved to 4°C and chilled for 30  
20 minutes. Texas-Red conjugated transferrin (Jackson Immunological) is diluted in cold serum-free media to a final concentration of 25 mg/ml. Transferrin is then bound to cells by incubating at 4°C for 45 min (Daro, E. *et al.* (1996) Proc Natl Acad Sci USA 93: 9559-9564). The temperature is raised to initiate endocytosis, and the tracer chased for 10 min (for early endosome) or 60 min (for the pericentriolar recycling endosome). As  
25 an alternative approach, Rab 11 (another marker for recycling endosome), is used for colocalization studies in both fixed and live cells. In addition, N-terminally fused DsRed-CFTR is used to examine its colocalization with markers described above, both on fixed cells and using the TILL VisTrack system for dynamic analyses in live cells. Using the TILL technology (Tvarusko, W. *et al.* (1999) Proc Natl Acad Sci USA 96:  
30 7950-7955), 500 profiles or vesicles are monitored in a single cell.

In accordance with the discoveries of the present invention, sialyltransferases are localized in TGN and cellubrevin-containing endosomes.

**B. Endocytosis in CF cells**

Initial experiments indicated that nonpolarized CF epithelial cells are competent for fluid phase endocytosis. Receptor-mediated endocytosis depends on a different mechanism than that of bulk fluid phase uptake although some aspects are shared (Li, G. *et al.* (1995) Proc Natl Acad Sci USA 92: 10207-10211). Receptor mediated endocytosis is investigated by the uptake of radioiodinated transferrin (Henkel, J. R. *et al.* (1998) Mol Biol Cell 9: 2477-2490). After native levels of transferrin have been depleted, CFT1 and corrected CFT1-LCFNS cells are incubated basolaterally with iodinated transferrin at 4°C. Receptor internalization is induced by warming up the cells. Filters are removed from the transwells at various time points, and activity counted to determine transferrin uptake (standardized against protein levels determined by BCA). Additional experiments are carried out with IB3-1 cells and derivatives, corrected for the fact that these cells are non-polarized. In accordance with discoveries of the present invention, endocytosis is impaired in CF cells.

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**C. Endosomal recycling affected in CF cells**

Since cellubrevin is primarily localized in the recycling endosome (Calvo, M. *et al.* (2000) J Biol Chem 275: 7910-7917), it is reasonable to assume that this compartment may have altered characteristics in CF cells, and specifically that recycling is altered in CF cells. It is contemplated that recycling is slowed down in CF (with important consequences on phagocytosis of bacteria, as is demonstrated subsequently), or, in the alternative, that recycling is increased or that redistribution of markers via recycling endosome is altered in CF vs. normal cells. Recycling in nonpolarized and polarized cells is examined and compared. For example, radioiodinated iron-saturated transferrin is added to the basolateral surface of epithelial cells and, following washes and receptor internalization, the kinetics of transferrin appearance in the medium on the apical and basolateral sides quantitated in CF vs. non-CF polarized cell lines (Henkel, J. R. *et al.* (1998) Mol Biol Cell 9: 247724-90). In accordance with the discoveries of the present invention, endosome recycling is affected in CF cells.

25  
30**D. Transcytosis in CF cells**

An important aspect of mucosal immunity depends on the deposition of antibodies across the mucosal surface into the lumen. It is contemplated that acidification of the TGN and endosomal network results in the misdirection or hindrance of transcytosis of

polymeric IgA from the basolateral surface to the apical surface. This is examined by measuring the transcytosis of radioiodinated IgA (Henkel, J. R. *et al.* (1998) Mol Biol Cell 9: 247724-90) across a polarized epithelial layer of CF (CFT1) or CFTR-corrected cells. Polarized CFT1 cells ( $R_t$ =about 100-200  $\text{cm}^2$  or higher) and CFTR corrected cells  
5 are incubated basolaterally with  $^{125}\text{I}$  IgA for 10 min at 37°C. After rinsing apical and basolateral surfaces, media is collected at successive time points, and the amount of radioactivity is determined. This permits an analysis of the kinetics of IgA transcytosis, and an investigation of whether IgA transcytosis is affected in CFTR-defective vs. CFTR-corrected cells. In accordance with discoveries of the present invention, IgA  
10 transcytosis is affected in CFTR-defective cells.

### EXAMPLE 8

#### **Interactions of *Pseudomonas aeruginosa* with respiratory epithelial cells and associated inflammation in the context of hyperacidification of intracellular compartments**

  
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The following experiments provide a connection between the effects of CFTR on acidification in intracellular compartments and infection and inflammation in CF. The results confirm the following three predictions: (i) Hyperacidification of TGN causes under-sialylation of glycoconjugates and thus can explain increased aGM1, and  
20 alterations of other surface glycolipids and proteins on CF cells. Decreased sialylation causes increased adhesion of *P. aeruginosa* and augments the downstream proinflammatory signaling. (ii) Decreased ingestion of *P. aeruginosa* by CF epithelial cells also occurs, as phagocytosis is affected in CF cells due to hyperacidification of the recycling endosome. As has been recently demonstrated, phagocytosis depends on the  
25 efficient flow of membrane to the nascent phagocytic cup, and maneuvers that interfere with the function of the recycling endosome prevent efficient phagocytosis (Bajno, L. *et al.* (2000) J Cell Biol 149: 697-706; Hackam, D. J. *et al.* (1998) Proc Natl Acad Sci USA 95: 11691-11696; and Cox, D. *et al.* (2000) Proc Natl Acad Sci USA 97: 680-785). (iii) Membrane signaling, including Toll-like receptors TLR2 and TLR4, are  
30 affected in CF cells due to the hyperacidified compartments involved in exocytosis and endocytosis. The resident time or distribution of these proinflammatory signaling receptors are affected in CF cells and contribute to increased production of proinflammatory cytokines and/or slower return to the baseline level in CF.

**A. *Pseudomonas aeruginosa* adhesion**

*P. aeruginosa* adheres better to CF than normal cells (Davies, J. C. *et al.* (1997) *Am J Respir Cell Mol Biol* 16: 657-63; Imundo, L. *et al.* (1995) *Proc Natl Acad Sci USA* 92: 3019-3023) due to altered glycosylation of surface receptors. Hyperacidification of TGN in CF cells represents the basis for this phenomenon. Adherence of *P. aeruginosa* to epithelial cells is determined after incubation of GFP expressing *P. aeruginosa* (Davies, D. G. *et al.* (1998) *Science* 280: 295-298) with CF vs. corrected cell lines. Non-adherent bacteria are removed by washing and separation in a percoll gradient and adherence to epithelial cells assayed by flow cytometry and independently by plating bacteria.

The interactions are further investigated by incubating the epithelial cells with specific blockers or competitors. Antibodies to aGM1 (Imundo, L. *et al.* (1995) *Proc Natl Acad Sci USA* 92: 3019-3023) (Waco Biochemicals, Richmond, VA) and lectins that bind to glycoconjugates are used to inhibit specific sites for *P. aeruginosa* adhesion. As another level of control, binding of piliated and nonpiliated *P. aeruginosa* PAO1 (the PAO1 strain that has been sequenced has a mutation in *pilC* and therefore is non-piliated) is compared. A large collection of well-characterized CF isolates (Boucher, J. C. *et al.* (1997) *Infect. Immun.* 65: 3838-3846) are used in additional experiments.

**B. *P. aeruginosa* uptake by epithelial cells**

It has been reported that *P. aeruginosa* is not taken up by CF epithelial cells as efficiently as by normal cells (Pier, G. B. *et al.* (1996) *Science* 271: 64-67). The phagocytosis by epithelial cells and subsequent desquamation have been proposed as a clearance mechanism by which bacteria are removed from the lung. Although this model has not been directly experimentally addressed, there are precedents in support of this possibility such as *Escherichia coli* clearance in the bladder (Mulvey, M. A. *et al.* (1998) *Science* 282: 1494-1497). The molecular mechanism for the reduced phagocytosis of *P. aeruginosa* by CF cells is at best controversial. Dr. Pier and colleagues have proposed that CFTR acts as a direct receptor for *P. aeruginosa* adhesion and subsequent uptake by the host cells (Pier, G. B. *et al.* (1997) *Proc Natl Acad Sci USA* 94: 12088-12093; and Gerceker, A. A. *et al.* (2000) *Infect Immun* 68: 861-870).

However, in accordance with the discoveries of the present invention, the reduced uptake is explained by the inefficient flow of membrane from the hyperacidified cellubrevin endosome to the points of bacterial entry. This explanation is confirmed by

assessment of the uptake of *P. aeruginosa* based on standard protocols (Pier, G. B. *et al.* (1996) *Science* 271: 64-67). After incubation of CF vs. non-CF cell lines with *P. aeruginosa*, extracellular bacteria are removed by washing and gentamicin treatment. Internalized bacteria are assayed by plating. In an alternative, FACS analysis is used  
5 with GFP expressing *P. aeruginosa*. Intra- and extracellular bacteria are differentiated by using antibodies against *P. aeruginosa* and fluorescently (red) labeling extracellular bacteria, which result in both green and red fluorescence vs. internalized organisms which results in only green fluorescence. It is contemplated that CF cells (for example IB3-1 and CFT1) internalize *P. aeruginosa* less efficiently than do the corrected cell  
10 types (for example, C-38 and CFT1-LCSFN). Correcting organellar pH (as described subsequently) restores bacterial ingestion efficacy in CF cells, in accordance with the discoveries of the present invention.

**C. Downregulation of proinflammatory receptors by endocytosis is deficient in CF  
15 cells**

In accordance with the discoveries of the present invention, hyperacidification and any downstream defects in the endocytic pathway affect signaling and proinflammatory cascade. These observations are based upon an examination of TLR2 and TLR4  
receptors.

20 The presence of TLR2 has been demonstrated on respiratory epithelial cells (Diamond, G. *et al.* (2000) *Immunol Rev* 173: 27-38). Toll-like receptors are part of the innate immune response to bacterial products and can act in conjunction with receptors such as CD14 to activate host defense genes (Diamond, G. *et al.* (2000) *Immunol Rev* 173: 27-38). TLR4 has been proposed to primarily respond to lipopolysaccharides of  
25 gram negative bacteria (Underhill, D. M. *et al.* (1999) *Nature* 401: 811-815). TLR2 has been implicated as a receptor for bacterial lipoproteins (Aliprantis, A. O. *et al.* (1999) *Science* 285: 736-739). Signaling from TLR2 and 4 activates a transduction cascade that results in translocation of NF- $\kappa$ B from the cytoplasm to the nucleus where it acts as a transcriptional factor activating proinflammatory cytokines. An explanation for the  
30 excessive inflammatory response observed in CF pulmonary tissues is that downregulation by endocytosis of TLRs or other proinflammatory receptors occurs improperly. If extended signaling through receptors occurs (in other words due to inefficient endocytosis or recycling defects), it could contribute to the over-exuberant inflammatory response in CF.

This explanation is confirmed by examining the kinetics of internalization of TLR2/4 by surface biotinylation approach (Aguilar, D. J. , Knudson, W. and Knudson, C. B. (1999) *Exp Cell Res* 252: 292-302; and Heda, G. D. and Marino, C. R. (2000) *Biochem Biophys Res Commun* 271: 659-564). Surface proteins of IB3-1 cells or CFT1 cells and their CFTR-corrected derivatives are biotinylated with a reducible form of biotin (NHS-SS-biotin). Cells are exposed to TLR ligands, either in the form of *P. aeruginosa* bacilli or more specific ligands such as LPS for TLR4 and the synthetic bacterial lipoprotein Pam3CysSerLys4 for TLR2. Epithelial cells are stimulated for 4 h and at additional time points. Biotin on proteins still remaining on the surface is removed by reduction with glutathione (or alternatively MESNA). The biotin on internalized proteins is not accessible to membrane-impermeant reducing agents. Cell lysates are incubated with streptavidin-coated agarose beads, and bound products analyzed by Western blotting using antibodies specific for TLR2 and TLR4. In this manner, downregulation of TLRs in CFTR-defective vs. CFTR-corrected cells is compared. In an alternative approach, the TLR ligands are modified by radiolabeling the substrates and their internalization monitored, and pro-inflammatory receptors other than TLRs are examined.

#### D. NF- $\kappa$ B activation

Proinflammatory signaling is also examined. In this approach, both TLR-dependent and TLR-independent NF- $\kappa$ B activation are monitored. This activation is studied in cells which have been serum and growth factor deprived for 16 hours. Observations are directed to whether hyperacidification in CF (and its normalization by chemical and other treatments; see subsequent Examples) affects the net NF- $\kappa$ B output. Cells are exposed to LPS, whole organisms, Pam3CysSerLys4, or TNF- $\alpha$ . Three methods are applied: (i) NF- $\kappa$ B nuclear translocation is analyzed by EMSA. Nuclear and cytoplasmic extracts are prepared using NE-PER nuclear and cytoplasmic extraction kit (Pierce). Labeling of oligonucleotides and EMSA gel shift are carried out as described in manufactures manual (Gel Shift Assay System E3050, Promega). (ii) A dual-luciferase assay system (Promega) is used to study NF- $\kappa$ B activation (Aliprantis, A. O. *et al.* (1999) *Science* 285: 736-739). An NF- $\kappa$ B-luciferase construct (pNF- $\kappa$ B-Luc; Clontech) expressing a firefly luciferase gene under control of an NF- $\kappa$ B consensus binding site luminesces in response to NF- $\kappa$ B activation. A second plasmid (pRL-SV40) is used for co-transfections with pNF- $\kappa$ B-Luc to normalize readouts. pRL-SV40

expresses the Renilla luciferase independent of NF-kB that can be measured in the same extract after quenching firefly luciferase. (iii) For immunofluorescence microscopy, cells are fixed, permeabilized, and NF-kB stained using monoclonal antibody against NF-kB p65 and goat-anti-mouse Texas-Red-labeled secondary antibody. Preliminary  
5 experiments showed a clear and significant difference between untreated/unstimulated cells (cytosolic staining) and human TNF-a stimulated cells (nuclear staining). (iv) In an alternative, activation of NF-kB is visualized in live cells, via a destabilized GFP under control of the NF-kB consensus binding site (pNFkB-d2EGFP; Clontech). Using live microscopy, the NF-kB activation is monitored in real time and differences in response  
10 times observed when comparing CF vs. corrected cell lines. The use of destabilized GFP is important, as constitutive non-specific accumulation of stable GFP could obscure induction and downregulation (Li, X. *et al.* (1998) *J Biol Chem* 273: 34970-34975). In accordance with the discoveries of the present invention, NF-kB is activated by the hyperacidification of the TGN and endosomes.

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#### **E. IL-8 production**

IL-8 levels resulting from activation of the inflammatory response in CF and normal cells is also measured. IL-8 levels from supernatants of cells exposed to proinflammatory ligands are assayed using an ELISA kit (Quantikine; R&D Systems,  
20 Minneapolis, MN). A comparison between IL-8 levels of CF and non-CF cells is made and a contribution of TLR activation assessed by using blocking antibodies specific to TLR2 and TLR4. In accordance with the discoveries of the present invention, normalizing pH in CF cells (described in subsequent Examples) reduces IL-8 levels or response duration or both.

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### **EXAMPLE 9**

#### **Normalization of the pH in intracellular compartments can correct interactions with *P. aeruginosa* and inflammation**

Hyperacidification of intracellular compartments in CF provides an important  
30 connection between the CFTR defect and infection and inflammation in CF. Moreover, this phenomenon presents a unique and relatively simple opportunity to correct this defect by normalizing the pH of relevant compartments using simple means such application of an alkalinizing agent, such as weak bases or additional, more sophisticated molecular tools to correct defects in TGN and endosomes. Use of pHluorin



GFP technology allows precise titration and adjustment of organellar pH to normal values.

**A. Normalization of pH with ammonia and chloroquine**

5 C-38 and CFT1 cells are transfected with cellubrevin-pHluorin GFP or TGN38-pHluorin GFP, and then incubated in media supplemented with 0-10 mM NH<sub>4</sub>Cl or chloroquine for 48 h at 37°C followed by ratiometric pH determination in TGN and endosomes. These weak bases cross membranes as unprotonated molecules and get protonated and trapped in acidic intracellular compartments while neutralizing acid.  
10 Initial results with low concentrations of NH<sub>4</sub>Cl indicated that hyperacidification of cellubrevin-labeled endosomes and TGN in CFTR mutant cells can be corrected to almost normal values compared to CFTR corrected cells, and that lectin binding patterns can be normalized by such treatments, as described previously. These titration results are confirmed and extended to additional weak bases, including but not limited to  
15 bicarbonate, with CFT1 and C-38 cells, and the results then applied to untransfected primary CF lung epithelial cells.

Additional analyses with weak bases using C-38, CFT1 and primary CF cells include: (i) Effects on *P. aeruginosa* adhesion and uptake by CF cells by normalizing the pH of the TGN and endosomes, using approaches already described in preceding  
20 Examples. It is contemplated that correction of pH in TGN and endosomes reduce *P. aeruginosa* binding and repair bacterial uptake by CFTR mutant cells, in accordance with discoveries of the present invention. (ii) Inflammatory response (using NF-κB and IL-8 as readouts) upon normalizing the pH of the TGN and the endosomes. The experimental approaches for testing NF-κB activation and IL-8 production will follow  
25 procedures outlined in Example 8. It is contemplated that correction of pH in TGN and endosomes reduce inflammatory response upon normalizing the pH of the TGN and the endosomes, in accordance with discoveries of the present invention. (iii) Effects on defects in fundamental processes of receptor-mediated endocytosis, endosomal recycling, or IgA transcytosis; it is contemplated that, in accordance with the discoveries  
30 of the present invention, such defects are corrected by pH normalization.

### B. Correction of pH defects using influenzae M2 protein

Influenza M2 protein is an acid-activated ion channel capable of increasing the pH of compartments in which it is located based on the protonation state of a transmembrane histidine residue (Henkel, J. R. *et al.* (1998) *Mol Biol Cell* 9: 2477-5 24790). It has been shown that M2 is primarily found in the apical plasma membrane, apical recycling endosomes and trans-Golgi network. M2-containing adenovirus strains are transduced into CFTR mutant cell lines. Transduction of IB3-1 and CFT1 cells with M2-adenovirus is carried out as previously described (G72). Briefly, the adenovirus is added to TGN-38- and cellubrevin-pHluorin GFP transfected (or alternatively co-10 transduced) cells at a multiplicity of infection between 120 and 6,000 for 1-2 h. The cells are incubated overnight to express M2 protein, and pH determined in TGN and endosomes using the standard pHluorin GFP methods as described previously. The effects of pH normalization are determined using assays as described above including lectin binding experiments. It is contemplated that correction of the intraorganellar pH 15 by M2 protein will result in decrease in infection and inflammation of respiratory CF epithelial cells, in accordance with discoveries of the present invention.

### C. Pharmacological agents

Based upon the preceding Examples, the effects of pharmacological agents to 20 decrease intracellular organelle pH are observed. One such class of pharmacological agents is clinically used inhibitors of  $H^+$  ATPase (for example omeprazole). The gastric proton pump inhibitors lansoprazole and omeprazole reverse acidification in CFTR mutant cells, in accordance with the discoveries of the present invention. However, one problem associated with these compounds is the fact that they were designed to 25 accumulate in parietal cells of the stomach (Suzuki, K. *et al.* (2000) *FEMS Microbiol Lett* 182: 69-72.). Another problem is that these compounds have been shown to inhibit the swelling-dependent chloride channels (ICISwell) (Schmarda, A. *et al.* (2000) *Br J Pharmacol* 129: 598-604). However, slight chemical modifications and appropriate dosing of these compounds allow targeting to bronchial epithelial cells, and eliminate 30 the undesirable inhibitory effect on chloride conductance.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been

described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be

5 within the scope of the following claims. Although contemplated mechanisms and hypotheses of the invention may be discussed, it is not necessary to understand any mechanism or hypothesis in order to use the present invention, and it is not intended that the present invention be so limited.

## CLAIMS

We claim:

1. A method of treating a cell, comprising:
  - (a) providing a cell comprising a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation, and
  - (b) exposing the cell to an alkalinizing agent.
  
2. A method of treating a cell, comprising:
  - (a) providing a cell comprising a CFTR gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes; and
  - (b) exposing the cell to an alkalinizing agent.
  
3. A method of treating a cell, comprising:
  - (a) providing a cell comprising a CFTR gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes; and
  - (b) exposing the cell to an alkalinizing agent, under conditions sufficient to decrease the hyperacidification.
  
4. A method according to Claim 3, wherein the decrease is at least about 0.2 pH units.
  
5. A method according to Claim 3, wherein the decrease is at least about 0.2 pH units but less than about 0.6 pH units.
  
5. A method according to Claim 3, wherein the hyperacidification is decreased to levels observed in a cell in which the CFTR gene mutation has been corrected.
  
6. A method according to Claim 3, wherein the cell is *in vivo*.
  
7. A method according to Claim 6, wherein the cell is a lung epithelial cell.

8. A method according to Claim 6, wherein the cell is a tracheal epithelial cell.
- 5 9. A method according to Claim 3, wherein the alkalinizing agent is a weak base.
- 10 10. A method according to Claim 9, wherein the weak base is selected from the group consisting of  $\text{NH}_4\text{Cl}$ , chloroquinone, and bicarbonate.
11. A method according to Claim 3, wherein the alkalinizing agent is a pharmacological agent, wherein the pharmacological agent perturbs cellular function to inhibit or decrease acid production in the trans-golgi network or endosomes or both, or to promote base formation in the trans-golgi network or endosomes or both, such that pH of the trans-golgi network or endosomes or both is increased.
- 15 12. A method according to Claim 11, wherein the pharmacological agent is selected from the group consisting of lansoprazole, and omeprazol.
- 20 13. A method according to Claim 12, wherein the pharmacological agent is selected from the group consisting of derivatives of lansoprazole and omaprazole; wherein the derivatives are effective to decrease the hyperacidification.
- 25 14. A method according to Claim 3, wherein the alkalinizing agent is an exogenous factor introduced by a heterologous gene, wherein the factor acts to inhibit or decrease acid production in the trans-golgi network or endosomes or both, or to promote base formation in the trans-golgi network or endosomes or both, such that pH of the trans-golgi network or endosomes or both is increased.
- 30 15. A method according to Claim 3, wherein the alkalinizing agent is added at a low concentration.

16. A method according to Claim 3, wherein the low concentrations range from about 0.01 to 0.1 mM.
17. A method according to Claim 3, wherein the alkalinizing agent is present in a form selected from the group consisting of a liquid, a vapor, a mist, a solid, and a powder.
18. A method of treating a cell, comprising:
- (a) providing a cell comprising a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation which results in hyperacidification of the cell organelles comprising trans-golgi-network and cellubrevin-endosomes;
  - (b) transforming the cell with a gene such that expression of the gene results in a decrease of the hyperacidification; and
  - (c) growing the cell under conditions effective to express the gene.
19. A method according to Claim 21, wherein the gene encodes an influenzae M2 protein.
20. A method of treating cystic fibrosis in a subject having cystic fibrosis, comprising administering a therapeutically effective amount of an alkalinizing agent.
21. A method of treating cystic fibrosis in a subject having cystic fibrosis, comprising administering an effective amount of an alkalinizing agent under conditions effective to result in a decrease of hyperacidity of trans-golgi network or endosomes or both within cells expressing a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation.
22. A method according to Claim 21, wherein the alkalinizing agent is administered to lung tissue of the subject.

23. A method according to Claim 21, wherein administering the alkalizing agent is effective to increase sialyltransferase in cells expressing a CFTR (cystic fibrosis transmembrane conductance regulator) gene mutation.
- 5
24. A method according to Claim 21, wherein administering the alkalizing agent is effective to decrease infection of lung tissue by pathogenic bacteria.
- 10
25. A method according to Claim 21, wherein administering the alkalizing agent is effective to decrease inflammation of the lung tissue.
26. A method according to Claim 21, wherein the alkalizing agent is present in a form selected from the group consisting of a liquid, a vapor, a mist, a solid, and a powder.
- 15
27. The method of Claim 21, wherein said administering is intranasally.
28. The method of Claim 21, wherein said administering is through respiratory inhalation.
- 20